T cell affinity and autoimmunity:

What is the origin of autoimmune T cells?

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Prof. Dr. Jörg Schibler

"Nothing in life is to be feared, it is only to be understood.
Now is the time to understand more, so that we may fear less."

Marie Curie
(1867-1934)

to my parents

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Abbreviations

aa	amino acid
Ab	Antibody
APC	Allophycocyanine
APCs	Antigen Presenting Cells
APC-Cy7	Allophycocyanine-Cyanine dye 7
APL	Altered Peptide Ligand
A700	Alexa Fluor 700
BB rat	Bio-Breeding Rat
BHI	Brain Heart Infusion
CD	Cluster of Differentiation antigen
CFU	Colony Forming Units
cTEC	cortical Thymic Epithelial Cell
CTL	Cytotoxic T Lymphocyte
DC	Dendritic Cell
DMEM	Dulbecco's modified eagle medium
DNA	Deoxyribonucleic Acid
DP	Double Positive ($CD4^+CD8^+$)
FACS	Fluorescence Activated Cell Sorting
FCS	Fetal Calf Serum
FITC	Fluorescein isothiocyanate
ITAM	Immunoreceptor Tyrosine Activation Motif
K_D	Dissociation Constant
mAb	monoclonal Antibody
MFI	Mean Fluorescence Intensity
mTEC	medullary Thymic Epithelial Cell
NOD	Non-Obese Diabetic Mouse
PAMPs	Pathogen associated-molecular patterns
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PE	Phycoerythrin
PE-Cy7	Phycoerythrin-Cyanine dye 7
PerCP	Peridinine Chlorophyll Protein
pMHC	peptide/Major Histocompatibility Complex
RAGs	Recombination-activation genes
RT	Room Temperature
RPMI	Roswell Park Memorial Institute 1640 Medium
PRR	Pattern Recognition Receptor
SA	Streptavidin
SP	Single Positive ($CD4^+$ or $CD8^+$)
TCR	T Cell Receptor
Tcra	T cell receptor α locus
Tcrb	T cell receptor β locus
tg	transgenic
TLR	Toll like receptor
T1D	Type 1 Diabetes (Autoimmune Diabetes)
OD	Optical Density

Summary

T cell receptor affinity for self-peptide/MHC ligand has a well established role in thymic selection. Above a certain affinity threshold ($K_D \leq 6$ μM) a developing $CD8^+$ T cell is negatively selected, while below this threshold it is positively selected, and no binding results in die by neglect. How TCR affinity contributes to the induction of autoimmune pathology remains poorly understood.

In the first part of my thesis, I examined the role of TCR affinity in peripheral T cells activation and the maintenance of peripheral tolerance. To do this, I make use of the RIP-OVA experimental autoimmune diabetes model. In RIP-OVA mice, OVA peptide (SIINFEKL) is under the control of the rat insulin promoter (RIP-OVA) and is expressed in both the pancreas and the thymus. Diabetes can be induced in RIP-OVA mice following adoptive transfer of OVA-specific $CD8^+$ T cells (OT-I T cells) and immunization with OVA peptide and lipopolysaccharide (LPS). To determine the role of TCR affinity in diabetes induction, mice were immunized with OVA peptide variants that span the threshold between positive and negative selection. Only mice receiving high affinity peptides (above the threshold for inducing negative selection) were able to induce diabetes. These findings were confirmed by histology which revealed highly infiltrated pancreatic islets only in mice immunized with above threshold ligand. In addition, higher affinity ligands induced stronger proliferation, activation marker upregulation and MAP kinase activation. Increasing the immunization dose of below threshold ligand was not sufficient to induce disease although additional experiments confirmed that below threshold activated T cells were not anergic. These findings indicate that the affinity threshold established in the thymus is maintained in the periphery. In addition, we identified asymmetric division as a mechanistic link between TCR affinity and the induction of autoimmune pathology. T cell priming with above-threshold ligand promotes sustained contact with antigen presenting cells (APCs), which promotes strong T cell polarity and asymmetric cell division. Asymmetric division results in the generation of “proximal” daughter cells capable of undergoing sustained proliferation and differentiation into short-lived effector T cells. High-affinity TCR-pMHC interactions also promoted T cell upregulation of VLA-4, an integrin important for infiltration into target tissue. In

contrast, T cells activated by below-threshold antigens underwent symmetric division, leading to abortive clonal expansion and failure to fully differentiate into tissue-infiltrating effector T cells.

In the second part of my thesis, I addressed the origin of self-reactive T cells. I was particularly interested to understand the impact of TCR affinity on 1) the efficiency of negative selection in the thymus; 2) the priming of a pathologic T cell response; and 3) the ability of a T cell to destroy self-antigen expressing target cells. To address these questions I made use of newly generated transgenic mouse lines expressing OVA-variant proteins with altered affinities for the OT-I TCR under the rat insulin promoter (RIP-variant mice).

To assess the efficiency of negative selection in RIP-variant mice, I crossed them with OT-I TCR transgenic mice. All double transgenic mice developed severe diabetes early after birth, suggesting that there were too many OT-I T cells in this system. As an alternative approach, I generated radiation bone marrow chimeras (further addressed below).

To assess the role of TCR affinity in the priming of a pathologic T cell response, I needed a model in which the affinity for priming is variable. I adoptively transferred OT-I T cells into RIP-variant transgenic mice followed with *Listeria monocytogenes* expressing OVA (Lm-OVA) (in the case of transfer into RIP-OVA mice) or variant peptide (depending on variant expression in host mice). Using this approach I found that T cells activated with threshold and below threshold ligands are poor inducers of effector T cell differentiation, and therefore comprise a low risk for autoimmunity.

To investigate the impact of TCR affinity for target tissue antigen on the induction of autoimmune pathology, I used a model in which priming efficiency is strong but the antigen affinity of the target tissue is variable. OT-I T cells were adoptively transferred into RIP-OVA variant mice and challenged with Lm-OVA. Diabetes was strongly induced in mice expressing both high affinity and threshold antigens in the pancreas. In contrast, mice expressing below threshold ligand remained diabetes free even after high numbers of OT-I T cells were transferred (10^7). These data indicate that threshold and below threshold target cells comprise a low risk for autoimmunity.

Since it was not possible to study T cell tolerance by breeding double transgenic mice, I generated mixed bone marrow chimeras. Lethally irradiated RIP-OVA and RIP-OVA variant mice were reconstituted with a mixture of OT-I and B6 bone marrow cells. These mice could be further used to determine the role of antigen affinity in negative selection, peripheral T cell priming and the ability of a T cell to lyse target cells. The results of these studies demonstrated that the efficiency of negative selection *in vivo* dramatically increases with above threshold self-antigens, such that fewer OT-I T cells survive negative selection in RIP-variant mice expressing high affinity antigen. By further challenging these mice with self-antigen/LPS, I was able to investigate whether OT-I T cells that survived negative selection were capable of inducing diabetes. Mice expressing antigens just above the negative selection threshold exhibited the highest risk of developing experimental autoimmune diabetes upon immunization with the corresponding peptide self-antigen. In contrast, mice expressing the cognate antigen for OT-I (OVA) (and the lowest of number of peripheral T cells) were completely free of diabetes, underscoring the importance of negative selection in preventing the accumulation of peripheral self-reactive T cells. In summary, these data suggest that just above the affinity threshold for negative selection, sufficient numbers of self-reactive T cells can escape deletion thereby constituting an increased risk for the development of autoimmunity.

Introduction

1 Innate and Adaptive Immunity

The immune system is composed of the innate and adaptive (acquired) immunity. The innate immune response acts early, it can distinguish self from non-self in a limited way. The innate immune system is composed of soluble factors (complement system) and cells of the innate immune system expressing pattern-recognition receptors (PRRs) that recognize common features of pathogens, so called pathogen-associated-molecular patterns (PAMPs). In most cases, phagocytic cells (macrophages, dendritic cells) that reside in the tissues internalize invading pathogens.

Individual cells of the adaptive immune system can specifically recognize pathogens by antigen specific receptors. It can eliminate those pathogens that overcome innate immunity, and it has the ability to provide enhanced protection against reinfection (immunological memory). The two main lymphocytes of adaptive immunity are B cells, which develop in the bone marrow and T cells, which develop in the thymus (see chapter 3). B cells secrete antibodies that kill extracellular pathogens (humoral response), whereas T cells are specialized to kill intracellular pathogens and to support the humoral response. Cells of the adaptive immune system recognize pathogens in a specific way, which is based on clonal selection of lymphocytes bearing antigen-specific receptors. Each lymphocyte carries cell-surface receptors of a single specificity, generated by the random recombination of variable receptor gene segments and the pairing of distinct variable chains. This process generates a self-tolerant T cell and a B cell repertoire with specificities against virtually all pathogens. The interplay between innate and adaptive immunity is important, because components of innate immunity provide instruction that enables the adaptive immune response to choose the right strategies for the successful elimination of pathogens (Bach and Chatenoud, 2011).

2 T cell receptor complex and signaling

The T cell receptor (TCR) complex is generated during T cell development in the thymus and TCR signaling is crucial for the induction of self-tolerance and later for T cell activation in the periphery. The TCR complex is made up of variable proteins for antigen-recognition and invariant proteins for signaling. The part in charge of antigen-recognition consists of two transmembrane glycoprotein chains, an α- and a β-chain, each containing a conserved and a variable region. The two TCR chains make a non-covalent association with a group of invariant proteins (CD3 γ, δ, ε and TCR ζ). TCR ζ and the intracellular domains of CD3 contain ITAMS (immunoreceptor tyrosine-based activation motifs), which are responsible for intracellular signaling (van Oers, 1999).

Upon engagement of TCR with peptide MHC complex (pMHC) on an APC, the co-receptor (CD8 or CD4) stabilizes the binding between TCR and pMHC. The first molecule to be recruited to the TCR-CD3 complex is LCK (a member of the SRC family kinase), which coupled to the co-receptor and comes into close proximity to phosphorylate the immunoreceptor tyrosine-based activation motifs (ITAMs) located on the CD3 -γ, -δ, -ε and -ζ chains (Fig.1). Phosphorylation of the ITAMS enables the recruitment of ZAP70 (ζ-chain associated protein kinase of 70 kDa), its phosphorylation by Lck and its activation. Activation of ZAP70 leads to the phosphorylation of four key tyrosine residues of the linker for activation of T cells (LAT). This recruits numerous signaling molecules forming the multiprotein complex termed the LAT signalosome. Important molecules that form this complex are phospholipase Cγ1 (PLCγ1), interleukin-2-inducible T cell kinase (ITK), NCK1, VAV1. The growth factor receptor-bound protein 2 (GRB2), GRB2-related adaptor protein GADS, SLP76 (SH2 domain-containing leukocyte protein of 76 kDa), and the adhesion- and degranulation-promoting adaptor protein (ADAP). The LAT signalosome propagates signals that can activate three major signaling pathways: The Ca^{2+}, the mitogen-activated protein kinase (MAPK), and the nuclear factor-κB (NF-κB) signaling pathway. The result of these pathways is the mobilization of transcription factors that are critical for gene expression and essential for T cell growth and differentiation. Signals propagated from the TCR also result in actin

reorganization and the activation of integrins, which promote cell adhesion (Brownlie and Zamoyska, 2013).

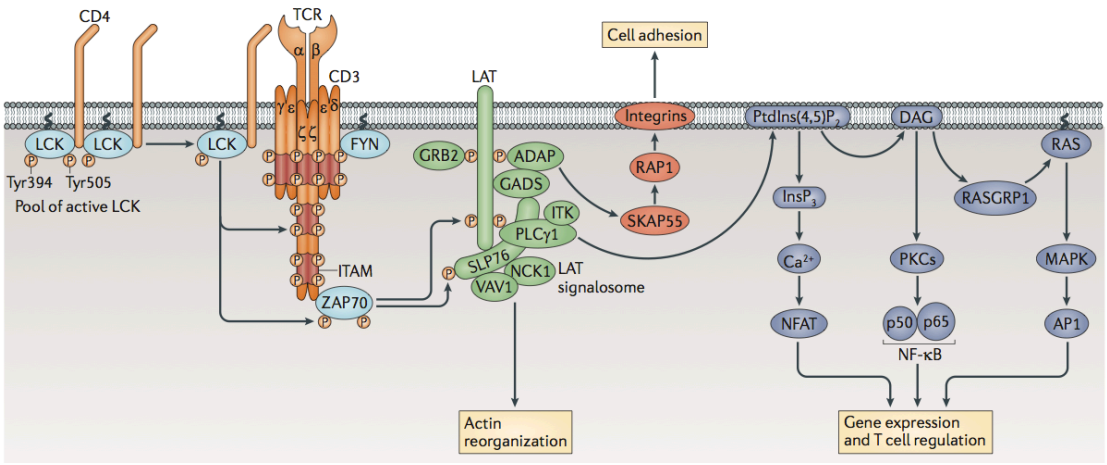

Fig. 1. Overview of the TCR complex and signaling cascades. The TCR is composed by an α- and a β-chain heterodimer, that specifically recognizes peptide in the context of MHC. The intracellular ζ-chains of the TCR and the CD3 complex (composed by CD3γ, CD3δ and two CD3ε chains) carry ITAMs. Upon engagement of the TCR-CD3 complex with pMHC, the coreceptor brings LCK close to the ITAMs and phosphorylation can occur. Phosphorylation of the ITAMs enables the recruitment of ZAP-70 and gets phosphorylated and activated by LCK. Activated ZAP-70 phosphorylates LAT and numerous signaling molecules are recruited (see text), which form a multiprotein complex (LAT signalosome). The LAT signalosome propagates signal which can activate three major signaling pathways, the The Ca^{2+}, the mitogen-activated protein kinase (MAPK), and the nuclear factor-κB (NF-κB) signaling pathway, which leads to the recruitment of transcription factors that are critical for gene expression and essential for T cell growth and differentiation. AP1, activator protein 1; DAG, diacylglycerol; $InsP_3$, inositol-1,4,5-trisphosphate; NFAT, nuclear factor of activated T cells; PKC, protein kinase C; PtdIns(4,5)P_2, phosphatidylinositol-4,5-bisphosphate; RASGRP1, RAS guanyl-releasing protein 1; SKAP55, SRC kinase-associated phosphoprotein of 55 kDa. (Brownlie and Zamoyska, 2013).

3 T cell Development and Central Tolerance

A protective and self-tolerant T cell repertoire is generated in the thymus, this is often referred to as central tolerance (Palmer, 2003; Starr et al., 2003; Stritesky et al., 2012; Werlen et al., 2003).

The thymus provides an optimal microenvironment for T cell development. Upon interaction with Notch1 receptor, which is expressed in the thymus, thymic emigrants commit to the T cell lineage (Wendorff et al., 2010). T cell differentiation is IL-7 dependent at the very immature stages and at the mature T cell stages IL-7 is crucial for survival and homeostatic proliferation (Ceredig and Rolink, 2012).

Developing thymocytes have to pass several stages (Fig.2) which is accompanied by a movement of thymocytes trough the different compartments of the thymus (Fig.3) before they are released into the periphery as functional T cells (Hogquist et al., 2005)

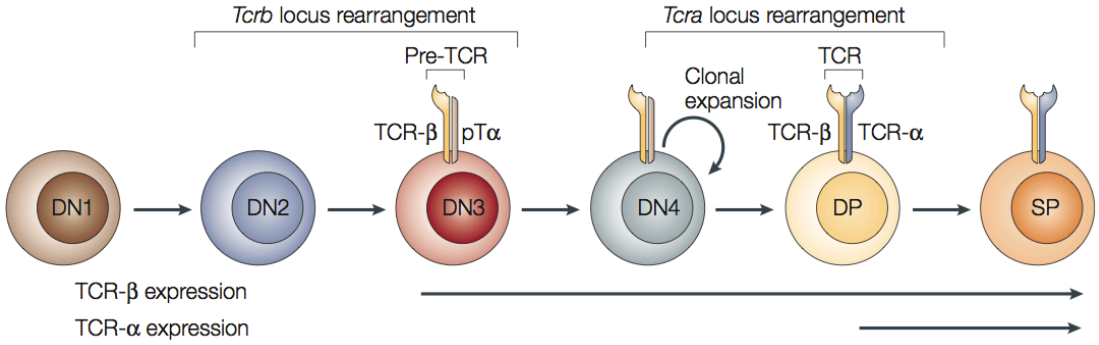

Fig. 2: Stages of T cell development. During T cell development, the recombination-activating genes (RAGs) are expressed and thymocytes in the DN2 or DN3 stage undergo rearrangement at the TCR β-chain locus (*Tcrb*), which is accessible at this stage. When a productive TCR β-chain is created, it pairs with the invariant pre-TCR α-chain (pTα), forming the pre-TCR. Signaling over the pre-TCR leads to further differentiation towards the DN4 and DP stages. During this transition the *Tcrb* locus becomes inaccessible and thymocytes undergo several rounds of proliferation. As the cell progresses to the DP stage, the *Tcra* locus gets accessible and rearrangement at the TCR α-chain occurs until successful rearrangement takes place. Finally, the αβ-TCR heterodimer is formed on the surface of DP thymocytes and ready for thymic selection. Adapted from (Hogquist et al., 2005)

During T cell development, thymocytes commit to αβ-, γδ-, or NK- T cell lineage. In this thesis the development of αβ- T cells is discussed. A discussion of the development of regulatory T cells and γδ-T cells is beyond the scope of this work.

In Fig.2 the movement of T cell progenitors (purple circles) trough the thymus is shown. T cell precursors from the bone marrow continually seed the thymus, they enter trough blood vessels near the cortico-medullary junction. The thymus is composed of endothelial cells that form an inner part (medulla) and an outer part, the cortex. Most steps of T cell development take place in the cortex, whereas thymocytes at the end of their development are mainly found in the medulla. The stages of T cell development are characterized by several surface markers, of most importance is the expression of the co-receptors CD4 and CD8 (Klein et al., 2009).

The earliest time point in the development of a thymocyte is the $CD4^-CD8^-$ Double Negative stage (DN). DN1 cells are found in the cortex, express Kit and are $CD44^+CD25^-$.

From the DN2 ($CD44^+CD25^+$) to the DN3 ($CD44^{low}CD25^+$) stage thymocytes migrate to the sub-capsular zone of the cortex and express the recombination-activation genes (RAGs) which enables them to undergo VDJ (variable, diverse, and joining) gene segment rearrangements at the T cell receptor (TCR) β-chain locus. First, D_β gene segments rearrange to J_β gene segments (this happens from the DN1 to DN2 stage), which is followed by V_β gene segments rearranging to the rearranged DJ_β gene segments at the transition from the DN2 to DN3 stage. Thymocytes that fail to make a productive rearrangement at the β-chain can be rescued by further rearrangements, because there are up to 52 V-, 2 D- and 13 J- gene segments in the human TCR β locus (Rowen et al., 1996).

When a productive TCR β-chain is created, further β-chain gene rearrangement is blocked (Abbey and O'Neill, 2008), thymocytes proceed to the DN3 stage where the TCR β-chain pairs with the invariant pre-TCR α-chain, forming the pre-TCR. Through the pre-TCR a thymocyte receives the signal for further differentiation and proceeds to the DN4 ($CD44^-CD25^-$) stage and the Double Positive (DP) stage.

At the transition from DN4 to DP stages, thymocytes undergo rigorous clonal expansion. After some rounds of division, proliferation stops and thymocytes start to rearrange the genes at the locus encoding the TCR α-chain. The α-chain TCR

rearrangement chooses from around 70 V- and 61 J segments for recombination (Rowen et al., 1996). There can be successive rearrangements of the T cell α-chain and it does not stop until positive selection or cell death intervenes.

Many different productive rearranged TCR α-chains can associate with a functional TCR β-chain, giving rise to T cells with ≈10^{18} different TCR specificities.

DP cells randomly move through the cortex and presumably scan cortical thymic epithelial cells (cTECs; Fig.2 blue cells) (Bhakta et al., 2005; Bousso et al., 2002; Ebert et al., 2008; Klein et al., 2009) for positively selecting ligands (see section 3.1). Most DP cells (around 90%) do not recognize self-peptide/MHC with their TCR, they receive no survival signal and die by neglect. Around 5% of DP thymocytes successfully generate a self-tolerant TCR and proceed to the Single Positive state (SP), undergoing CD4 or CD8 lineage commitment, before they are released into the periphery (Palmer, 2003).

Thymocytes need to distinguish between self and non-self in order to generate immunological tolerance. Therefore, positively selected SP cells migrate to the medulla where self-antigens are presented on mTECs and bone marrow derived DCs to thymocytes for their TCR functionality and self-reactivity. SP cells that bind self-peptide too strongly with their TCR (around 5%) are deleted from the T cell repertoire by a process called negative selection (see section 3.1). In summary, positive selection takes place in the cortex, whereas negative selection of tissue-restricted antigens takes place in the medulla. However, although thymic medulla provides a specialized environment for negative selection, it has been shown that negative selection on ubiquitous self-antigens can also take place in the cortex (McCaughtry et al., 2008).

The development of a thymocyte takes about 4-5 days (Klein et al., 2009) and bone marrow reconstitution assays showed that the generation of a functional T cell cell (from the entry of a T cell progenitor into the thymus until the exit to the periphery as a functional T cell) takes approximately 4 weeks.

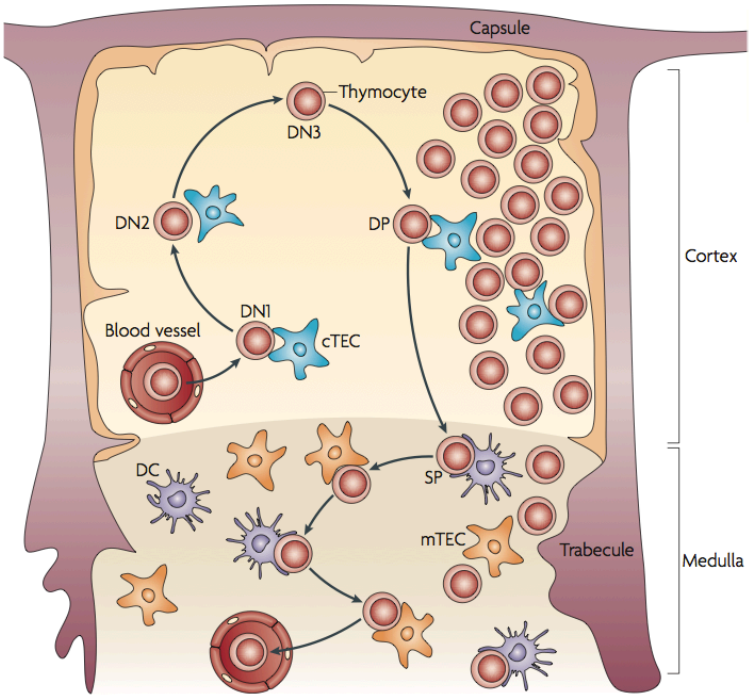

Fig. 3. Overview of T cell developmental stages, migration trough thymus and interaction with stromal cells. T cell progenitors enter the thymus close to the cortico-medullary junction. The earliest developmental stage of thymocytes is the CD4-CD8- double negative (DN) state. During the development from DN1 to DP, cells migrate towards the sub-capsular zone of the cortex and the TCR β-chain is rearranged and a pre-TCR is formed (DN3). After several rounds of division, the TCR α-chain is rearranged and upon successful rearrangement, a TCRβ-chain associates with a TCRα-chain, forming the TCR at the double positive stage (DP). DP scan cortical thymic epithelial cells (cTECs) for self-antigens and if they bind within a certain affinity range, DP cells undergo CD4 or CD8 lineage commitment which leads to single positive (SP) thymocytes. SP cells rapidly relocate to the medulla, where they scan medullary antigen-presenting cells (mTECs and DCs) where deletion of thymocytes, which bind to strong self-antigen with their TCR sorts out autoreactive T cells (see section 3.1), generating a self-tolerant TCR repertoire (Klein et al., 2009).

3.1 Positive and Negative Selection

Central Tolerance ensures that T cells that are potentially self-reactive are deleted from the T cell repertoire. This requires the expression of the huge set of self-antigens of the body in the thymus. Soluble antigens are transported by the blood flow to the thymus whereas the delivery of tissue-restricted antigens (TRAs) is not as simple. Several cell types are involved in the presentation of TRAs, bone marrow derived dendritic cells, cortical thymic epithelial cells (cTECs) and medullary thymic epithelial cells (mTECs). The protein AIRE is highly active in mTECs and drives the

ectopic expression of TRAs (Anderson et al., 2002), thus generating perfect conditions for negative selection in the medulla. The importance of Aire is underlined by the induction of multi-organ autoimmune disease in humans when Aire-deficient mTECs have reduced levels of TRAs on their surface (Anderson et al., 2002; Taniguchi et al., 2012).

The underlying mechanism that promotes the survival of a T cell (positive selection) or apoptosis of self-reactive T cells (negative selection) is dependent on the binding strength (affinity) between a TCR for self-peptide/MHC complex (Fig.4a) (Palmer and Naeher, 2009). If the TCR of a double positive thymocyte binds very weak to self-peptide/MHC the developing T cell is neglected, it does not receive any survival signals trough the TCR and will die (Fig.4b).

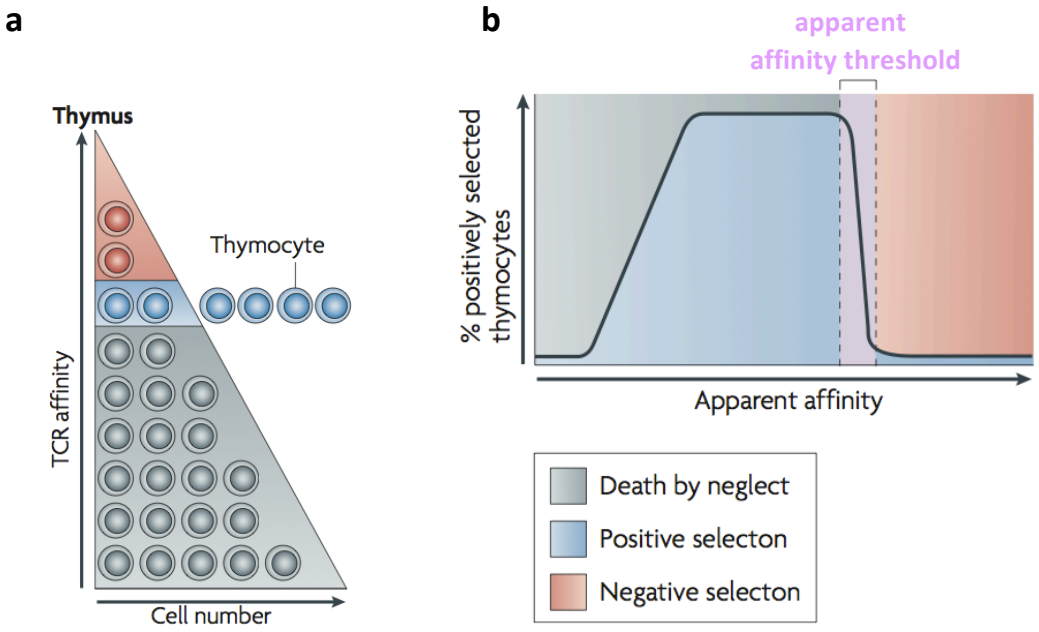

Fig. 4. Thymic selection depends on the TCR's affinity for self-peptide MHC complexes.
a) Most developing thymocytes express a TCR which does not bind to self-peptide MHC complexes and therefore do not receive a survival signal and die by neglect (grey). Low-affinity binding of TCR with self-peptide MHC induces survival and differentiation (known as positive selection). Positively Selected thymocytes are released in the periphery (blue). High-affinity binding of TCR with self-peptide MHC induces apoptosis, a process which is known as negative selection (red).
b) The outcome of thymic selection depends on the strength of interaction (affinity) between TCR and self-peptide MHC complex. For CD8 T cells, there is an apparent affinity threshold ($K_d \leq 6$ μM, indicated in pink).
(Palmer and Naeher, 2009)

If the TCR binds with moderate affinity to self-peptide/MHC, the developing T cell gets positively selected and receives a survival signal. In contrast, the TCR of a self-reactive T cell binds with high affinity to self-peptide/MHC and gets negatively selected. Studies with three MHC-I restricted transgenic TCR lines defined an affinity threshold for the binding of TCR to self-peptide/MHC at a $K_D \leq 6$ µM where negative selection is initiated (Daniels et al., 2006; Naeher et al., 2007). MHC-I restricted thymocytes binding self-antigens with a lower affinity ($K_D > 6$ µM) undergo positive selection.

Although central tolerance prevents autoimmune T cells from entering the peripheral repertoire, negative selection is not a perfect process and a small number of high affinity, self-reactive T cells enter the periphery (Taniguchi et al., 2012).

The fact that there is such a sharp affinity threshold distinguishing positive and negative selection indicates that the TCR has an astonishing precision in reading antigen affinity and a remarkable flexibility in signaling divergent thymocyte fates.

Two models have been proposed to describe how this differential signaling occurs, the occupancy model and the kinetic proofreading model. The occupancy model suggests that the level of TCR occupancy determines selection outcome. This model is antigen-concentration dependent, which is based on the observation that strong ligands at extremely low doses lead to positive selection (Ashton-Rickardt et al., 1994; Sebzda et al., 1994). However, in many cases the lymphocytes generated in this way developed into regulatory T cells ($CD4^+$ Tregs or $CD8\alpha\alpha^+$ T cells) (Leishman et al., 2002a; Sakaguchi, 2005). The kinetic proofreading model suggests that the TCR measures how long an antigen interacts with a TCR (half life); low affinity antigens have short TCR interactions, leading to a weak signal, whereas higher affinity antigens have longer interactions with the TCR and initiate a strong signal (Daniels et al., 2006; Goldstein et al., 2004; McKeithan, 1995). Indeed, experiments that are used to measure affinity (see section 3.3) illustrate the correlation among peptide affinity, half-life and selection outcome (Daniels et al., 2006; Davis et al., 1998; Williams et al., 1999).

3.2 Signaling molecules involved in negative selection

In both positive and negative selection, molecules of the MAPK pathways (p38 and JNK) are involved. The difference between positive and negative selection signaling seems to be a matter of kinetics and location of the intracellular signaling mediators. In studies with DP thymocytes, high-affinity ligands (negative selector) induced an intense and transient burst of extracellular-signal-regulated kinase (ERK) activity, whereas low affinity ligands (positive selectors) induced ERK slowly and over a prolonged period of time (Mariathasan et al., 2001; Werlen et al., 2003; Werlen et al., 2000). Nevertheless, ERK activation in thymocytes seems paradoxical, since one would expect negative-selecting, high-affinity ligands to interact longer with the TCR (compared to positive-selecting ligands), yet they activate ERK only briefly. Furthermore, Daniels et al. found that a negative selecting ligand (Q4R7) of pre-selection OT-I double-positive thymocytes induced more rapidly and quantitatively higher Ca^{2+} flux compared to the positive selecting ligand Q4H7 (Daniels et al., 2006). Daniels and al. further showed that above threshold antigens induced a strong early peak in LAT, p23- zeta, and ZAP-70 phosphorylation, whereas below threshold antigens induced weak phosphorylation of these molecules and the peaks were delayed. Sometimes, these differences are very small and the question arises how thymocytes can read such small changes. The spatial compartmentalization of key molecules helps thymocytes to choose between negative or positive selection; Strong interaction between pMHC and TCR leads to the localization of phosphorylated ZAP-70 and LAT at the cell membrane, where it results in the activation of the ERK signaling cascade. In contrast to that, below threshold antigens are weak recruiters of these signaling molecules at the plasma membrane, most molecules remain in the cell plasma, resulting in weaker and delayed signaling (Daniels et al., 2006).

In summary, positive and negative selection share many signaling molecules. Nevertheless, the completely different outcome (survival in positive selection and apoptosis in negative selection) probably comes from the order of activation and localization of signaling molecules, this determines which transcription factors are triggered and this ultimately controls the selection outcome. Finally, how the TCR

regulates the same molecules distinctly in positive vs. negative selection remains an open question. Prasad et al. did computer simulation and experiments, which suggest that the contrast between positive and negative selection originates in the ability to differently activate Ras proteins (Prasad et al., 2009).

3.3 Measurement of TCR-pMHC Affinity

The binding strength between two molecules is termed affinity. The term avidity is often used to describe the overall strength of a binding when multiple molecules are involved. Three molecules are involved when TCR together with a coreceptor forms a docking site for a monomeric pMHC ligand. Therefore, the term apparent affinity may be most suitable (Naeher et al., 2007).

T cells translate the physical binding of TCR, coreceptor and pMHC into a cellular response. In line with this, both bio-physical and biological parameters can be used to describe how well a TCR responds to pMHC; Bio-physical parameters describe the kinetic aspects of monomeric interactions between two molecules, i.e. TCR/pMHC affinity, on- and off-rates, and half-life times of the complex. These parameters can be measured by surface plasmon resonance (SPR, BiaCore).

The main components of this system are a sensor chip with gold film, a flow channel, a light source and a detection system. One of the interacting molecules is immobilized onto the sensor surface (i.e. pMHC molecule) and the second molecule is in solution (i.e. TCR) (Axelrod and Omann, 2006). However, SPR is unable to resolve the contribution of the coreceptor (Garcia et al., 1996).

The photoaffinity labeling system overcomes the limits of SPR (Luescher et al., 1994). For this method, pMHC that carries a photoreactive azidobenzoic acid (ABA), which is linked to an amino acid in the peptide, can be used (Naeher et al., 2007). After specific binding of pMHC monomers to the corresponding T cell, photoactivation of the ABA group results in cross-linking of pMHC to the TCR. This allows quantitative analysis of pMHC monomer binding (Cebecauer et al., 2005; Hudrisier et al., 1998; Kessler et al., 1997; Naeher et al., 2007).

Another method to assess the physical strength of TCR and pMHC is the use of soluble pMHC tetramers (Altman et al., 1996; Wang and Altman, 2003). Using FACS, quantitative analysis of tetramer binding, the off rate, and half-life can be assessed by measuring mean fluorescence intensity (MFI) of the pMHC tetramers that dissociate from the TCR over time.

There are other factors besides physical parameters that influence the binding strength between TCR and pMHC; or signals received over the TCR can translated differently into a cellular response (Fahmy et al., 2001; Slifka and Whitton, 2001). In this setup, T cell responses are measured when different amounts of pMHC ligands are present. This measurement is usually referred to as functional avidity, the readout usually is cytokine production (i.e. IFN-γ production). Of note, functional avidity measurements correlate well in most cases with the physical parameters (Zehn et al., 2012).

Most TCR-pMHC binding kinetic studies have been analyzed three-dimensionally (3D) in solution. Since close contact between TCR and APC is required to trigger a signal, where TCR and pMHC are anchored on the membranes of the apposing cells, this contact is of two-dimensional (2D) nature. Compared to 3D measurements in solution, 2D data have faster kinetics and higher affinities, as a result of a large (about 100-fold) increase in the association rate. Furthermore, the off rate (k_{off}) of the pMHC to the TCR was also increased, but not as much as the association rate (k_{on}) (Huang et al., 2010; Huppa et al., 2010). This suggests that T cells use rapid antigen sampling and serial engagement of a few strong pMHCs by TCRs out of a large self-antigen pool to assure deletion of self-reactive T cells in the thymus. The study of Lillemeier et al. goes in line with these observations (Lillemeier et al., 2010). In naïve T cells, TCR and the key adaptor molecule of the TCR signaling pathway LAT, exist in separate domains of the membrane (protein islands). Upon T cell activation, these domains form a big cluster (immunological synapse), thus creating a perfect environment for amplifying TCR-pMHC interactions.

3.4 Peripheral Tolerance

Central tolerance is imperfect, and some self-reactive T cells enter the periphery. Nevertheless, autoimmune diseases are rare, indicating that additional mechanisms maintain tolerance in the periphery (Peripheral Tolerance). Three mechanisms of peripheral tolerance are described in the literature (Redmond and Sherman, 2005):

1) Ignorance
2) Deletion or Anergy
3) Suppressive T cells (regulatory T cells, Tregs)

T cell ignorance describes a T cell that does not get activated upon pMHC presentation by APCs. In the absence of a pathogen, a T cell encounters self-antigen and is not activated because of the lack of co-stimulatory molecules on the APC and the absence of inflammatory cytokines.

The deletion model proposes that antigen encounter induces apoptosis in a T cell. Zinkernagel and coworkers found that a single dose of antigen (GP peptide of LCMV) resulted in priming of GP-specific CD8 T cells, whereas multiple immunizations resulted in tolerance (Aichele et al., 1995). Deletion of self-reactive T cells is observed in the presence of chronic antigenic stimulation, whereas anergy (a state of unresponsiveness) is induced upon strong TCR stimulation (Redmond et al., 2005).

Regulatory T cells (TRegs) are CD4+ T cells expressing CD25, the IL-2 receptor and the transcription factor FoxP3. TRegs have been shown to play key roles in the maintenance of self-tolerance and negative control of a variety of physiological and pathological immune responses. The depletion of the regulatory T cell compartment can result in the activation of self-reactive T cells, leading to autoimmune diseases (Maloy and Powrie, 2001; Sakaguchi et al., 1995; Shevach, 2000; Singh et al., 2001; von Boehmer and Daniel, 2013).

4 T cell Activation

4.1 General aspects of CD8 T cell activation

T cells that emigrate from the thymus are naïve, they have not encountered any foreign antigen before. With the blood, they circulate to secondary lymphoid organs like spleen and lymphnodes. Because circulating naïve T cells cannot enter peripheral non-lymphoid tissue, the foreign tissue derived antigens (i.e. after a viral infection) have to be transported to the T cells in the lymphoid organs. There, professional antigen presenting cells (APCs) like B cells and dendritic cells (DCs) that have taken antigen up, process it and present the antigen on their surface in the context of MHC class I or II to CD8 and CD4 T cells, respectively (Jenkins et al., 2001).

When a CD8 T cell forms a stable interaction with an APC, the T cell gets activated (primed). A naïve CD8 T cell needs 3 signals to become an effector T cell: (1.) specific TCR stimulation, (2.) co-stimulation and (3.) cytokine signal (Brownlie and Zamoyska, 2013).

The first signal is generated when the TCR makes a specific interaction with pMHC presented by an APC. The second signal is delivered via co-stimulatory molecules located on APCs, which ensures an effective immune response. Known co-stimulatory molecules that interact with their counterparts on T cells are CD40-CD40L and CD80,86-CD28.

The early activation marker CD69 is a type II transmembrane glycoprotein with a C-type lectin binding domain and its expression is rapidly induced upon priming of naïve T cells. Although a specific ligand for CD69 has not been identified, its wide distribution on T cells and its' capacity of inducing intracellular signals made it a reliable marker for early T cell activation.

Upon activation, T cells start to proliferate. They receive the proliferation signal over IL-2. IL-2 binds the IL-2 receptor (CD25) located on the T cell. CD25 is upregulated shortly after CD69. In order to follow up proliferation, T cells are labeled with a dye (i.e. CFSE), which gets diluted by every division. Proliferation is a useful readout to identify an immunological relevant epitope in an immune response, since antigen-reactive clones accumulate in sufficient amounts for their analysis.

When there is an infection, inflammation triggers TNF-α production and upregulates VCAM-1, a cell adhesion molecule expressed on the vascular epithelium. Activated T cells express VLA-4, a ligand for VCAM-1, which mediates the adhesion and infiltration of cytotoxic T cells into the target tissue where they clear the pathogens.

The initial contact of T cells with its' target cell is mediated by unspecific adhesion molecules. Endothelial cells and immune cells express ICAM-1 that is making contact with LFA-1 on the T cell. This binding allows the T cell to scan the surface of the target cell for the presence of specific pMHC complex. Specific signaling trough the TCR prolonges the binding, leading to a stable interaction for initiating lysis of the target cell. Cytotoxic T cells contain specialized lysosomes called lytic granules. When they release granules at the site of the target cell contact, they release granzymes and perforin, latter mediates target cell lysis by making pores in the membrane.

4.2 Asymmetric T cell division

It remains unclear how the immune response balances the generation of cytotoxic CD8 T cells (CTLs). Under certain conditions, many self-reactive CTLs are generated which can induce autoimmune diseases. This implicates that division must be asymmetric in order to confer disparate fates of the daughter cells. Several studies reported that T cells undergo asymmetric T cell division and that this form of cell fate choice is used to generate CD8 memory T cells (Chang et al., 2007; Oliaro et al., 2010).

In the first part of the results, the role of TCR affinity in asymmetric T cell division and generation of CTLs is discussed.

5 Murine Models for Autoimmunity

5.1 Transgenic mice as a model to study autoimmune diabetes

Transgenic mouse models became important tools to study autoimmune diseases. Most studies about autoimmune diabetes (type 1 diabetes, T1D) were performed with rodents that develop a spontaneous T1D, the bio-breeding (BB) rat and the non-obese diabetic (NOD) mouse. It has been showed that both, β- cell-specific autoantibodies and autoreactive T cells are involved in the disease, but that only T cells induce tissue pathology (Bach, 1994; Bendelac et al., 1987; Like et al., 1985).

The identification of a key antigen in T1D is an issue, since there seem to be more than one. Studies from NOD mice suggest the obvious candidate pro-insulin/insulin (B chain insulin peptide B:9-23) (Nakayama et al., 2005). Furthermore glutamic acid decarboxylase (GAD) (Baekkeskov et al., 1990), the tyrosine phosphatase (IA-2) (Atkinson and Maclaren, 1993; Kawasaki et al., 1996; Payton et al., 1995), the islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP) (Lieberman et al., 2003; Mukherjee et al., 2005), and the more recently described zinc transporter ZnT8 (Wenzlau et al., 2010).

In studies with NOD mice, CD3 antibodies lead to remission of the disease. The underlying mechanism is the induction of apoptosis in effector T cells, whereas the suppressive effect of Tregs is maintained or even increased (Belghith et al., 2003; Chatenoud, 2010; Chatenoud et al., 1997; Chatenoud et al., 1994)

Other mouse models were designed, where autoimmune diabetes was not spontaneous, but induced. Therefore, an antigen is cloned under the tissue specific rat insulin promoter (RIP) into the pancreatic β-cells of a mouse, which is normally not expressed by these cells. This “neo-self antigen” is accepted as self from the immune system. Upon the adoptive transfer of transgenic T cells expressing the specific TCR for the neo-self-antigen, they get activated and destroy insulin producing β cells, the hallmark of autoimmune diabetes. This principle is used to generate several mouse models for induced diabetes, i.e. RIP-gp33 (immunogenic peptide of LCMV) (Ohashi, 1991) and RIP-mOVA (immunogenic peptide found in chicken egg).

The RIP-mOVA mouse was generated in the lab of William Heath (Kurts et al., 1996). The membrane bound form of ovalbumin is expressed under the rat insulin promoter (RIP) in the β-cells of the pancreas, in the renal proximal tubular cells and in the testis of male mice. T1D induction in RIP-mOVA mice depends on the numbers of adoptive transferred OVA-specific $CD8^+$ T cells (OT-I). Coinjection of OVA-specific $CD4^+$ T cells (OT-II) was able to induce T1D when low numbers of OT-Is were transferred, that are usually not enough to elicit disease (Kurts et al., 1997). Since we use lower OT-I concentrations in our studies, this likely explains why we have to immunize with OVA peptide and an inflammatory stimulus like LPS or Listeria to induce T1D.

In contrast to the spontaneous T1D models, T1D in neo-self antigen transgenic mice is induced by T cells with a single TCR specificity and is thus a monoclonal system (Haskins and McDuffie, 1990; Katz et al., 1993; Verdaguer et al., 1997; Wong et al., 1996). In our studies we use the RIP-mOVA model and we are aware that this is an artificial system to study autoimmune diabetes. Several years ago our laboratory identified altered peptide ligands (APLs) of OVA where 1-2 amino acids were exchanged (see section 5.2). This leads to peptides with different affinities for the OT-I TCR. We generated transgenic mice expressing these variants under the RIP promoter. This gave us a novel tool with the unique advantage to study the role of T cell affinity in the development of autoimmune diabetes.

Currently, T1D in humans is treated by insulin injection that is accompanied by the regular surveillance of blood glucose (self-measurements by patients). Although insulin treatment allows patients to have a virtually normal lifestyle and life span, chronic insulin therapy has (over long term) great limitations, as it does not fully protect against degenerative complications of the disease.

In the attempt to restore self-tolerance in autoimmune diabetes, drugs targeting the cells of the immune system were evaluated and nicely reviewed in 2011 by Bach and Chatenoud (Bach and Chatenoud, 2011). It is important to mention that therapies are most effective at an early stage of disease, when there is still a sufficient amount of functional β-cells left to allow metabolic recovery.

Monoclonal antibodies to CD20 and CTLA4-Ig effectively dampen the autoreactive immune response, but they do not restore self-tolerance (Orban et al., 2011; Pescovitz et al., 2009). The same results were obtained with the immune suppressive drug cyclosporine, on top of that came adverse effects (i.e. nephrotoxicity) when cyclosporine was administered at higher doses (1988; Skyler and Rabinovitch, 1992).

So far, vaccination studies with soluble β-cell self-antigens were disappointing in clinical trials (Walter et al., 2009; Wherrett et al., 2011), which is complicated by the problematic of identifying key antigens.

Epidemiological data predict that the incidence of T1D will increase over the next years, it will affect more younger children within the first 5 years of life (Patterson et al., 2009). For obvious reasons, long-term complications linked to chronic insulin therapy will increase, underlining the importance of finding effective therapies for autoimmune diabetes, by combining strategies or by the discovery of new therapies.

5.2 Ovalbumin and APLs for the specific activation of T cells

Ovalbumin (OVA) is a protein found in the white part of chicken eggs. OT-I and OT-II T cells (both Vα2, Vβ5 TCR chain) are specific for OVA. OT-I T cells are $CD8^+$ and recognize the 8 amino acid (aa) long OVA epitope $SIINFEKL_{257-264}$ presented on MHC class I (K^b). $CD4^+$ OT-II T cells are MHC class II restricted and are specific for the 12 aa epitope $OVA_{323-339}$. Altered peptide ligands of OVA (APLs) are OVA variants with variable affinity. They were generated by exchanging aa's within the SIINFEKL epitope and specifically induce positive or negative selection in OT-I T cells (see Table 1). We found the below-threshold antigen Q4H7 (in blue) is inducing positive selection in OT-I thymocytes and is a weak activator of effector functions in peripheral OT-I T cells. T4 (in grey) is the threshold antigen since it induces positive or negative selection in thymocytes and can activate OT-I T cells in periphery inducing diabetes 25% in RIP-sOVA mice (see results part I). Q4R7 is the above-threshold antigen, it is a potent inducer of negative selection and a strong priming antigen in periphery leading to 100% diabetes (see results part I).

Table 1

[a] Affinity	[b] K_D (nM)	[c] Peptides	[d] AA Sequence	[e] Thymus	[f] Periphery
low	51 ± 9.1	Q4H7	SIIQFEHL	positive	-
	55 ± 10.1	T4	SIITFEKL	pos. or neg.	+/-
	48 ± 9.5	Q4R7	SIIQFERL	negative	+
high	3.7 ± 0.7	OVA	SIINFEKL	negative	+

(a) Affinity for OT-I TCR. (b) K_D values were determined from nonlinear regression analysis of tetramer binding curves with pre-selection double-positive thymocytes at 37°C. The ± value represents the 95% confidence interval from the nonlinear regression analysis (adapted from Daniels et al., nature, 2006)
(c-d) OVA and OVA variants and it's amino acid sequence are displayed. (e) Outcome of FTOC experiments (Daniels et al., nature, 2006). Positive and negative means positive and negative selection in the thymus, respectively. (f) Shows diabetes induction (+) or no diabetes induction (-) in RIP-OVA mice that were adoptively transferred with $5x10^6$ OT-I T cells and immunized with appropriate peptide and LPS (King et al., Immunity, 2012). AA Sequence, amino acid sequence.

5.3 LPS and *Listeria monocytogenes* as tools to activate innate immunity

T cells of the adaptive immunity do not recognize pathogens directly, they need cells of the innate immune system that present the foreign antigen in the context of MHC. Macrophages and dendritic cells (DCs) are professional antigen presenting cells. They carry a set of PRRs, i.e. toll like receptors (TLRs) on their surface, recognizing unique features of viruses and bacteria. TLR4 recognizes lipopolysaccharide (LPS), a molecule of the outer membrane of gram-negative bacteria. In this work we used LPS as an adjuvant for the immunization protocols. That means, that together with LPS we co-injected peptides. LPS activates DCs via TLR4, they upregulate MHC class I and II and co-stimulatory molecules and they become a potent APC presenting OVA to T cells.

Another approach to make DCs present the peptide of interest, is to infect a mouse with living bacteria that are forced to express the peptide of interest. Compared to a single LPS injection, the immunization with living bacteria confronts the immune system with many foreign-antigens, they are available in larger amounts and they remain longer in the host before the bacteria get cleared.

Listeria monocytogenes is a gram-positive bacterium and it can escape the phagolysosome of phagocytic cells and spreads from cell to cell without exposure to the extracellular milieu (Pizarro-Cerda et al., 2012). Therefore, the host reacts with a complex interplay between the elements of innate and adaptive immunity (Pamer, 2004). In the initial phase of infection, cells of the innate immune response like neutrophils, macrophages and NK cells control bacterial growth (Carr et al., 2011; Yin and Ferguson, 2009). At a later stage of infection, there is substantial evidence that CD8 T cells are crucial to clear the infection and to protect from reinfection with *Listeria monocytogenes* (Cook et al., 1999; Strehl et al., 2006). We and others have shown that *Listeria monocytogenes* is not able to activate transgenic T cells in an unspecific way to induce experimental autoimmune diabetes (Zehn and Bevan, 2006). In the laboratory, the bacterium is relatively easy to genetically manipulate and to grow. For our studies, we introduced the OVA variants (APLs) into the DNA of Listeria (Lm-Q4H7, Lm-T4, Lm-Q4R7 and Lm-OVA) and used it as a tool for

immunization in the mouse model. This makes it a good tool to study T cell activation.

6 Materials and Methods

6.1 Reagents

Reagent	Company, Country
Anti-Biotin MicroBeads	(Miltenyi, Germany)
Avidin/Biotin Blocking Kit	(Vector laboratories, UK)
BHI Agar	(Sigma, Switzerland)
BHI Broth	(Sigma, Switzerland)
Cryomold	(Sakura, NL)
Coverslips	(Menzel, Germany)
Cuvets, semi-micro, PS	(Ratiolab; Roche, Switzerland)
DMEM	(Gibco, UK)
Eosin (0.2%)	(Medite, Germany)
Erythrocyte lysis buffer	(BioLegend, UK)
FoxP3 staining kit	(eBioscience, Austria)
Heat inactivated Foetal Calf Serum (FCS)	(Amimed®, BioConcept, Switzerland)
KCl	(Fluka, Switzerland)
KH_2PO_4	(Fluka, Switzerland)
Live/dead (fixable) cell staining kit	(Invitrogen, UK)
Low-Tox Rabbit Complement	(Cedarlane, Canada)
LPS (lipopolysaccharide)	(Sigma, Switzerland)
MACS Separation Columns (LS)	(Miltenyi, Germany)
non-essential amino acids	(Gibco, UK)
$Na_2HPO_4*2H_2O$	(Sigma, Switzerland)
NOPIL	(Mepha Pharma AG, Switzerland)
Objektträger Thermo scientific	(Menzel, Germany)
OCT Embedding Matrix	(Cell Path, UK)
ParPen	(Cell Path, UK)
RPMI Medium 1640 (1x) with L-Glutamine	(Gibco, UK)
Sodium Pyruvate	(Gibco, UK)
Vectashield® Mounting Media with DAPI	(Vector laboratories, UK)

96 Well Cell Culture Cluster Round Bottom (Corning, Germany)

6.1.1 Solutions and media

Embedding solution		Glycerol
	10%	TRIS Buffer (0.1M, pH 7.4)
FACS buffer		PBS
	3%	FCS
Hybridoma cell media		RPMI
	10%	FCS
NOPIL		Drinking water (for mice)
	0.80 mg	Sulfametoxazolum
	0.16 mg	Trimethoprimum
1xPBS (pH 7.3)	137 mM	NaCl
	8 mM	$Na_2HPO_4*2H_2O$
	2.7 mM	KCl
	1.5 mM	KH_2PO_4
T cell medium		DMEM
	10%	FCS
	1x	non-essential amino acids
	1 mM	sodium pyruvate
	100 U/ml	penicillin
	100 µg/ml	streptomycin
	2 mM	L-glutamine
	1 mg/ml	gentamycin
	50 µM	β-mercaptoethanol

6.1.2 Antibodies

Antigen	**Clone**	**Provider**
Antibodies for IHC:		
α-CD8α (purified)	53-6.7	BD
α-CD45.1-biotin	A20	BioLegend
α-swine Insulin (guinea pig)		Dako
α-rat IgG A647		invitrogen
SA-A488		BD
α-guineapig IgG A555		invitrogen
Antibody containing Hybridoma supernatant for T cell depletion of bone marrow		
α-CD4 (rat IgM)	RL172	K. Hafen & G. Holländer
α-CD8 (rat IgM)	31M	K. Hafen & G. Holländer
α-Thy1.1 (rat IgM)	T24	K. Hafen & G. Holländer
α-Thy1.2 (rat IgM)	HO	K. Hafen & G. Holländer
Antibodies for flow cytometry analysis:		
CD3ε-APC	145-2C11	BD
CD3ε-biotin	145-2C11	BD
CD4-Alexa700	RM4-5	BD
CD4-biotin	RM4-5	BD
CD8α-biotin	53-6.7	BD
CD8α-PE-Cy7	53-6.7	eBioscience
CD8β-PerCP Cy5.5	53-5.8	BioLegend
CD11b-biotin	M1/70	BD
CD11c-biotin	HL3	BD
purified CD16/32 antibody	2.4G2	BD
CD44-APC	IM7	BioLegend
CD45.1-FITC	A20	BD

CD45.1-PE	A20	BD
CD45.2-APC-Cy7	104	BD
CD62L-PE-Cy7	MEL-14	BioLegend
CD122-FITC	TM-beta1	BioLegend
F4/80-biotin	BM8	eBioscience
FoxP3-A647 (APC)	150D	BioLegend
IFN-γ-PE	XMG1.2	BioLegend
Ly6C/G-biotin	RB6-8C5	BD
NK1.1-biotin	PK136	BD

6.1.3 Peptides

Lyophilized peptides were received from EUROGENTEC (Belgium).
Peptide stocks of 100 µM were kept at -70°C by dissolving peptides in fresh DMSO, hardly dissolving peptides were heated up until complete dissolving was achieved. $OVA_{323-339}$ epitope was dissolved in water.

MHC-I restricted 8 aa's long $OVA_{257-264}$ epitope
Sequence: NH_2- SIINFEKL-COOH Molecular Weight (MW) 963.15 g/l

MHC-I restricted $OVA_{257-264}$ variants (Altered peptide ligands)
Q4H7; sequence: NH_2- SIIQFEHL-COOH Molecular Weight (MW) 986.15 g/l
T4; sequence: NH_2- SIITFEKL-COOH Molecular Weight (MW) 950.15 g/l
Q4R7; sequence: NH_2- SIIQFERL-COOH Molecular Weight (MW) 1005.19 g/l

MHC-II restricted 17 aa's long $OVA_{323-339}$ epitope
Sequence: NH_2- ISQAVHAAHAEINEAGR-COOH MW 1774.9 g/l

6.1.4 Tools for Glucose measurements

DIABUR TEST® 5000 (ACCU-CHEK® product, Roche, Switzerland) for urine glucose measurements and Contour® sensor for blood sugar measurement (Bayer, Switzerland) using test strips for blood glucose measurements.

6.1.5 Instruments

Blood Glucose Meter Ascensia® CONTOUR®	Bayer, Switzerland
Cell counter Beckman Coulter Z2	Beckman Coulter, Germany
Cell Density Meter CO8000	WPA, UK
Confocal Microscope LSM 780	Zeiss, Switzerland
Flow cytometer BD FACSCanto II	BD, Germany
Irradiator Gammacell 40 Exactor	Best Theratronics, Canada
Laminar Flow Hood	Heraeus
Multifuge 3 S-R	Heraeus Holding GmbH, Germany
Table Top Centrifuge 5417R	Eppendorf AG, Germany
Water bath GFL	Faust, Switzerland

6.1.6 Software

Program	**Application**	**Provider**
FlowJo	FACS analysis	Tree Star Inc., USA
Prism	Graphs, calculations	Graph Pad Software Inc., USA
ImageJ	Image analysis	NIH, USA

6.1.7 Generation of OVA variant transgenic mice

The vector carrying the construct containing OVA (SIINFEKL) and human transferrin receptor (Tfr) was previously described (Gleeson, J. Biol. Chem., 1992) (Teasdale et al., 1992). In brief, the cDNA of Transferrin-Receptor-OVA protein (TfR-OVA) construct was inserted into the pBlue Vector (purchased from the laboratory of Gleeson) just before the Hind III site (Fig.5).

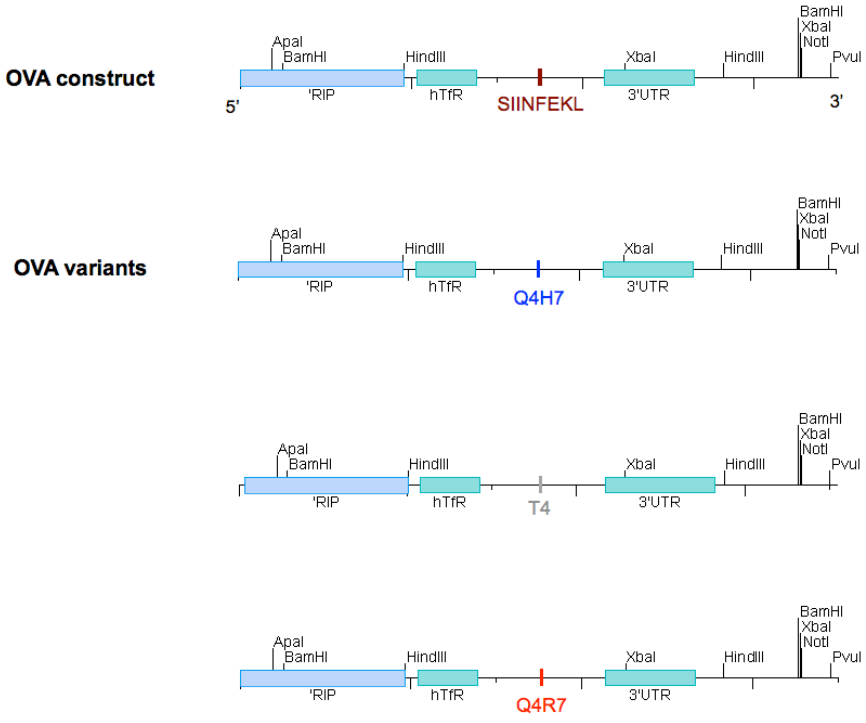

Fig. 5. Overview of the constructs used for the generation of RIP-OVA or RIP-OVA variant transgenic mice. The neo-self antigen (OVA or OVA variant) is under the control of the Rat Insulin Promoter (RIP). The introduction of the human transferrin receptor (hTfR) into the construct ensures that OVA or OVA variants are anchored to the the cell membrane of Langerhans β-cells in the pancreas.

Three different variants of the short region within the OVA cDNA were used to generate RIP-Q4H7 (SIIQFEHL), RIP-T4 (SIITFEKL), and RIP-Q4R7 (SIIQFERL) respectively. This was achieved by digesting the vector at the restriction sites Apa I, Not I and Pvu I, allowing a reliable purification of the 3.2 kB Apa I, Not I fragment containing the TfR-OVA or OVA variant mutation aimed for injection. The 3.2 kB fragment was injected into the pronucleus of fertilized C57BL/6 zygotes and

transplanted into NMRI foster mothers. Founder mice were obtained and selected founders were bred to C57BL/6 mice.

Selection of founders was based on following findings:

H7S7 and H7S5 express the neo-self-antigen in the pancreas since double transgenic mice (crossed to OT-I) exhibit lethal diabetes (see Results Part III, Fig. 3). In contrast to H7S7 mice, H7S5 mice exhibited strongly diabetes in adoptive transfer experiments with Lm-OVA infection (Results Part III, Fig. 4). Therefore, H7S5 was excluded.

In the double transgenic breeding of OT-I x founder T4S2 we observed no diabetes compared to the other founders (Results Part III, Fig. 3). This strongly suggests that T4S2 does not express the neo-self antigen in the pancreas and therefore was excluded.

In bone marrow chimera experiments, both Q4R7 founders deplete self-reactive OT-I T cells (Results Part III, Fig. 5) but R7Q2 founders were excluded because they developed spontaneously diabetes (transient diabetes).

6.1.8 Generation of double transgenic mice

RIP-OVA mice expressing a soluble form of ovalbumin, and RIP-mOVA, expressing a membrane-bound form of Ova under the control of RIP (King et al., 2012; Kurts et al., 1997), OT-I TCR transgenic mice recognizing $K^b/Ova_{257-264}$, and CD45-1 congenic C57BL/6 mice were all obtained from The Jackson Laboratory (Bar Harbor, ME).
Double transgenic mice were generated by crossing homozygous OT-I with homozygous RIP-sOVA, RIP-mOVA or RIP-mOVA variant transgenic mice. We assed the mortality by comparing the number of F1 pubs born and the number of F1 pubs surviving two weeks after birth. All animal work was done in accordance with the Federal and Cantonal laws of Switzerland.

6.2 Histochemistry

6.2.1 Preparation of frozen tissue sections

Pancreata were removed and frozen in biopsy holders (Cryomold® Biopsy, Sakura, Netherlands) containing OCT compound (Tissue-Tek®, Sakura, Netherlands). Sections of 6-8 µm were cut using a cryostat Microm HM 560 (Zeiss, Switzerland). After cutting, slides were air-dried overnight and then wrapped in a little plastic bag and stored at -20°C.
Prior staining, slides were slowly thawed at RT in order to avoid water condensation on the tissue.

6.2.2 Hematoxilin/Eosin (H&E) staining

Frozen tissue sections were air-dried and hydrated for 1min each in a decreasing ethanol series (95%, 80%, 75% ethanol, and water). Staining with Eosin (Sigma, Switzerland) was performed (1min incubation) followed by three washing steps in water (each wash for 1min). Then, staining for 50 seconds in Hematoxylin (Sigma, Switzerland) was performed, followed by three washing steps in water (each wash for 1min). Sections were finally dehydrated (1min in 75%, 80%, 95% ethanol) and coverslipped using embedding solution. Coverslip was sealed with nail polish to prevent drying of the embedded tissue.

6.2.3 Immunohistochemistry (IHC)

Detection of surface markers and intracellular insulin was performed on frozen tissue sections using specific antibodies. First, pancreas sections were fixed with cold aceton (stored at -20°C) for 5-7 min, followed by drying of the slides at RT. Since there were always three pancreas sections on one slide, a line around the sections with ParPen (Cell Path, UK) were drawn, allowing different stainings at the same time. Slides were put back into wash buffer for 3min to allow rehydration. Further processing of the slides were performed in a wet chamber to prevent drying. If incubation with biotinylated antibodies followed, endogenous biotin was blocked

using the Avidin D/Biotin Blocking Kit (Vector laboratories, UK). After blocking, slides were washed twice in wash buffer (2x 3min) with fresh changes of buffer, followed by staining with primary antibodies α-CD8α (rat), α-CD45.1-biotin, α-insulin (guineapig) and incubated for 1h at RT. After staining with primary antibodies, slides were washed twice in wash buffer (2x 3min) with fresh changes of buffer, followed by staining with secondary antibodies (α-rat-A647, α-guineapig-A555, SA-488) for 30min at RT. To determine the intensity of unspecific background staining, secondary antibodies were incubated for 1 hour at the same concentration as specific antibodies. Slides were subsequently coverslipped using Vectashield® with DAPI (Vector laboratories, UK) as mounting medium. Coverslip was sealed with nail polish to prevent drying of the embedded tissue.

6.3 T cell isolation from lymphnodes and B cell isolation from spleen

For T cell isolation, mice were sacrificed and lymphnodes of TCR transgenic Rg KO mice were dissected out and kept in T cell media on ice for further processing. Lymphnode suspensions were obtained by disrupting lymph nodes through a 70-µm nylon cell strainer (BD). To obtain B cells, spleen from B6 mice were disrupted in the same way and erythrocyte lysis was performed for 1min at RT (BioLegend, UK). B cells were enriched by untouched magnet beads isolation. Therefore, spleen suspension was incubated with α-F4/80-biotin (eBioscience, Austria), α-CD3-biotin, α-CD4-biotin, α-CD8α-biotin, α-NK1.1-biotin, , α-Gr-1-biotin, α-Ly6G/C-biotin, α-NK1.1-biotin, α-CD11c-biotin and CD11b-biotin (BD, Germany), and incubated for 30min at 4°C. After a washing step, α-biotin magnetic beads were added and run over a magnetic LM column according to the manufacturers instruction (Miltenyi, Germany). T and B cells were washed with T cell media prior cell counting. Single cell suspensions were counted using a Beckman Coulter Z2 particle counter (Beckman Coulter).

6.4 Adoptive T cell transfer and immunization with peptide/LPS

After isolation of T cells (see section 6.3) cell concentrations were adjusted in PBS containing 1% FCS and 200µl of cell suspension was intravenously (i.v.) injected into the tail vain of mice that were previously heated for 3min under a red lamp.
One day after adoptive T cell transfer, immunization (intraperitoneal injection) with OVA peptide or APLs together with LPS was performed. Peptides were pre-diluted in DMEM to 2 mM. Concentrations of peptides and LPS was adjusted to 50µg/mouse and 25µg/mouse, respectively. Peptides and LPS was diluted in 1xPBS and total injection volume was 200µl/mouse.

6.5 Tetramer, surface antibody staining and flow cytometry

Staining was performed in 96-well plate (Corning, Germany) using $3x10^6$ cells per well. K^b-OVA (SIINFEKL) tetramers were produced as previously described (Cebecauer et al., 2005; Daniels et al., 2006). Unspecific Fc receptor blocking was performed using purified CD16/32 antibody (BD) for 10 min at 4°C in PBS supplemented with 3% FCS (FACS buffer). After that, cells were washed and K^b-OVA tetramer staining was performed for 1h at 4°C in FACS buffer. Additional surface staining was performed (in the presence of tetramer) for 30min at 4°C in FACS buffer with the following antibodies: CD45.1-FITC, CD45.1-PE, CD45.2-APC-Cy7, CD8α-biotin, CD4-Alexa700, CD3ε-APC (all from BD) CD8β-PerCP Cy5.5, CD44-APC, CD122-FITC, CD62L-PE-Cy7 (all from BioLegend) and CD8α-PE-Cy7 (eBioscience). In a separate staining, intracellular staining of FoxP3 was performed (see intracellular cytokine detection) with the antibody FoxP3-A647 (BioLegend).

6.6 Intracellular cytokine detection

For the detection of intracellular cytokines, splenocytes from day-3-immunized RIP-OVA mice were stimulated with OVA peptide *in vitro* in the presence of monensin for 4 hours at 37°C. After surface stainig, intracellular staining was performed using the intracellular staining kit from eBioscience (FoxP3 staining kit). In brief, cells were

fixed and permeabilized for 20min on ice followed by a washing step with permeabilization buffer (1xPerm/Wash). In the same buffer, staining with α-IFN-γ was performed for 30min on ice, washed once and resuspended in FACS buffer prior analysis by flow cytometry.

6.7 Killing assay

For the assessment of cytotoxic T lymphocyte function in vivo, splenic B cells from CD45.1 congenic mice were labeled with 5 µM ($CFSE^{hi}$) or 0.5 µM ($CFSE^{lo}$) CFSE. $CFSE^{hi}$ and $CFSE^{lo}$ cells were pulsed for 4 hours at 37°C with 2 µM OVA or VSV peptide, respectively. Cells were mixed at a 1:1 ratio, and 10^7 APCs were injected intravenously into recipients harboring activated OT-I T cells. Surviving B cells in spleen and lymphnodes were determined after 5 hours by flow cytometry.

6.8 Culturing of *Listeria monocytogenes* and infections

Recombinant *Listeria monocytogenes* (Lm) expressing the full length OVA protein containing the CD8 epitope SIINFEKL (OVA) or altered ligands Q4R7, T4, or Q4H7 were previously described (Zehn et al., 2009b). Frozen stocks of these strains were grown in brain-heart infusion broth (Thermo Fisher Scientific) to mid log phase. Bacterial numbers were determined by measuring the OD at 600 nm. 5,000 cfu per mouse were injected intravenously in 0.9% NaCl (B.Braun).

6.9 Generation of radiation bone marrow chimeras

Recipient mice (CD45.2) were lethally irradiated with 900 rad (GammaCell, Best Theratronics, CA). Bone marrow cells were isolated from femurs and tibiae from 5-8 week old B6 (CD45.1) and OT-I Rg KO (CD45.1/2) mice. After red blood cell lysis (RBC lysis Buffer, BioLegend), cells were washed and filtered (70-µm cell strainer, BD). To deplete mature T cells, bone marrow cells were stained with anti-CD4, anti-CD8, anti-Thy1.1, anti-Thy1.2 (were obtained from hybridoma supernatants, kindly

provided by K. Hafen and G. Holländer) followed by incubation with complement (Low-Tox-M Rabbit Complement, Cedarlane, CA) at 37°C for 45 minutes. This step was repeated for OT-I bone marrow cells, using anti-CD8 antibody only. A 70% : 30% mixture of B6 and OT-I Rag KO bone marrow cells (3.5 x 10^6 total cells) were injected i.v. into lethally irradiated (900 rad) recipient mice. Mice were treated with antibiotics (Nopil, Mepha Pharma AG) for approximately 10 wk after irradiation. The congenic markers CD45.1 and CD45.2 were used to identify the T cells after the reconstitution.

6.10 Urine and blood glucose measurements

Urine glucose was assessed with test stripes from ACCU-CHEK (DIABUR-TEST 5000, ACCU-CHEK). Mice with sustained urine glucose levels >1000 mg/dl were considered diabetic. Blood samples from the tail vain were read on a Contour blood glucose reader (Bayer). Mice with blood glucose levels >15 mmol/l were considered to be hyperglycemic.

6.11 Data analyses

Flow cytometry measurements were performed on a FACSCanto II (BD) machine and the data were analyzed with FlowJo software (Tree Star). Graphs were generated with Prism (GraphPad Software). The horizontal line represents geometric mean. Where indicated, unpaired-t-test and One-way-ANOVA was performed (Prism, GraphPad Software).

Thesis Objectives

The biological mechanisms that lead to a breakdown of tolerance and the onset of autoimmune disease remain poorly understood. I want to know where autoreactive T cells originate and how the affinity of TCR for pMHC impacts the efficiency of negative selection, the ability to prime an autoimmune response and the ability of an autoreactive T cell to lyse a target cell expressing the auto-antigen.

Negative selection of autoreactive T cells in the thymus prevents the accumulation of strongly autoreactive T cells in the periphery. Nevertheless, central tolerance is an imperfect process, which allows for the escape of some self-reactive T cells into the periphery. In the second part of the results I will address the question of how TCR affinity for pMHC impacts the efficiency of negative selection.

In specific, I will analyze the effect of antigen affinity on T cell priming and the induction of autoimmune pathology (results part I). I would like to understand if the affinity threshold that was established in the thymus is maintained in periphery. If it is, I will further explore the mechanisms leading to the maintenance of peripheral tolerance in my system. I would like to clarify why below-threshold antigens are poor inducers of autoimmunity, whether below-threshold antigens induce anergy; and how a break in tolerance does occur. Further I would like to understand how above-threshold ligands prime T cells to cause autoimmune pathology.

Using transgenic mice that express pancreatic antigen with different TCR target affinities (results part II), I will also distinguish between the affinity necessary to prime an autoimmune response and the affinity necessary to lyse a target auto-antigen. In particular, I will establish the critical number of autoreactive T cells required to induce tissue pathology. Finally, I will put the results into context by describing the circumstances that are likely to result in the highest risk of developing an autoimmune disease.

7 Results and Discussion

7.1 Part I, Immunity Article

T Cell Affinity Regulates Asymmetric Division, Effector Cell Differentiation, and Tissue Pathology

Carolyn G. King, Sabrina Koehli, Barbara Hausmann, Mathias Schmaler, Dietmar Zehn and Ed Palmer. Immunity 2012

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Article

T Cell Affinity Regulates Asymmetric Division, Effector Cell Differentiation, and Tissue Pathology

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SUMMARY

The strength of interactions between T cell receptors and the peptide-major histocompatibility complex (pMHC) directly modulates T cell fitness, clonal expansion, and acquisition of effector properties. Here we show that asymmetric T cell division is an important mechanistic link between increased signal strength, effector differentiation, and the ability to induce tissue pathology. Recognition of pMHC above a threshold affinity drove responding T cells into asymmetric cell division. The ensuing proximal daughters underwent extensive division and differentiated into short-lived effector cells expressing the integrin VLA-4, allowing the activated T cell to infiltrate and mediate destruction of peripheral target tissues. In contrast, T cells activated by below-threshold antigens underwent symmetric division, leading to abortive clonal expansion and failure to fully differentiate into tissue-infiltrating effector cells. Antigen affinity and asymmetric division are important factors that regulate fate specification in CD8$^+$ T cells and predict the potential of a self-reactive T cell to mediate tissue pathology.

INTRODUCTION

Most T lymphocytes in an individual have a history of self-reactivity during positive selection in the thymus; they recognize proteins encoded in the major histocompatibility complex (self-MHC) and loaded with self-peptides, which are peptide fragments derived from the body's own proteins. Although positive selection promotes the development of T cells with weak self-reactivity, negative selection blocks the development of T cells with strong self-reactivity (Goldrath and Bevan, 1999b; von Boehmer et al., 1989). To generate a self-tolerant peripheral T cell repertoire, developing CD8$^+$ T cells use a specific affinity threshold for T cell receptor (TCR) binding to self-antigens to initiate negative selection (Daniels et al., 2006; Naeher et al., 2007). Although thymocytes respond to peptides over a wide range of affinities, the transition from positive to negative selection is extremely sensitive and occurs within an extremely narrow affinity range.

The kinetics of two-dimensional TCR-pMHC interactions are important for determining how a TCR signals (Huang et al., 2010). Efficient negative selection in the thymus prevents most self-reactive high-affinity T cells from entering the peripheral repertoire but ligands at the affinity threshold induce negative selection only when expressed at sufficient concentrations. Thus, it is clear that some T cells escape thymic selection and persist in the peripheral lymphoid organs. Although escaping T cells have a threshold affinity for their target antigen and do not generally cause spontaneous autoimmunity, disease can be induced by activation with cross-reactive foreign antigens (Gronski et al., 2004; Zehn and Bevan, 2006). TCR affinity for foreign antigens is clearly a factor in the induction of autoimmunity (Gronski et al., 2004), but how this affinity relates to the selection threshold is not known. In addition, recent work by Zehn, et al. has shown that T cells with weak self-reactivity can be induced to divide and acquire effector functions after bacterial infection (Zehn et al., 2009).

In this study, we wondered how much TCR affinity is necessary for autoimmune pathology and, specifically, whether the affinity threshold established during central tolerance induction plays a role in maintaining peripheral tolerance. We found that only higher affinity, "suprathreshold" antigens (above the threshold for negative selection in the thymus) are able to induce tissue pathology. Suprathreshold ligands promoted long-lasting T cell:antigen-presenting cell (APC) contacts required for T cell polarization and asymmetric T cell division, which generates CD8^{hi} proximal daughter and CD8^{lo} distal daughter cells (Chang et al., 2011; Chang et al., 2007). We further showed that proximal daughters exhibited prolonged binding to antigen-loaded APCs and that such prolonged binding resulted in sustained proliferation and differentiation of these daughter cells into short-lived effector cells (SLECs). In contrast, distal daughters underwent limited proliferation and had a reduced potential to induce tissue pathology. Finally, CD8$^+$ T cells activated by low-affinity, "subthreshold" ligands (below the threshold for negative selection in the thymus) primarily underwent symmetric division, resulting in the production of progeny with shorter APC conjugation and reduced differentiation into SLECs. Taken together, these data reveal that the establishment of T cell polarity is dependent on TCR affinity and is required for the full differentiation of T cell effectors capable of initiating tissue pathology.

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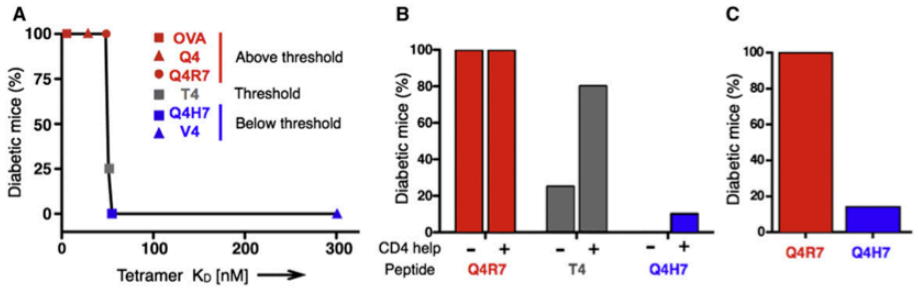

Figure 1. Incidence of Autoimmune Diabetes in RIP-OVA Mice after Immunization with High-Affinity OVA or Altered Peptide Ligands

(A) Diabetes was induced by adoptive transfer of 5×10^6 Rag-2-deficient OT-I cells into RIP-OVA mice and subsequent intraperitoneal immunization with 50 μg OVA peptide (SIINFEKL) or the indicated altered peptide ligands and 25 μg LPS (n = 5, OVA; n = 5, Q4; n = 11, Q4R7; n = 12, T4; n = 11, Q4H7; n = 5, V4). Mice were considered diabetic if urine glucose levels were ≥1000 mg/dl.

(B) Diabetes was induced as in Figure 1A with or without the addition of 1×10^6 allogeneic B6.C-H-2 bm12 B cells (n = 5, Q4R7; n = 10, T4; n = 11, Q4H7).

(C) Diabetes was induced by the adoptive transfer of 3×10^4 OT-I cells into RIP-OVA mice and subsequent infection with recombinant Lm-Q4R7 (n = 8) or Lm-Q4H7 (n = 14). (see also Figure S1). Representative data are shown of n ≥ 3 separate experiments.

RESULTS

TCR Affinity Regulates the Induction of Tissue Pathology

To determine the level of self-reactivity necessary for the induction of tissue pathology, we evaluated the role of TCR affinity in mediating autoimmune diabetes. RIP-OVA mice express ovalbumin in the β cells of the pancreatic islets under the control of the rat insulin promoter (RIP) (Blanas et al., 1996). Although adoptive transfer of high numbers of naive OT-I TCR transgenic $CD8^+$ T cells specific for K^b-OVA is insufficient to induce disease, immunization with OVA peptide after OT-I T cell transfer results in rapid β cell destruction and diabetes (Behrens et al., 2004). After adoptive transfer of OT-I T cells and immunization with OVA peptide or various peptide variants (Figure S1A in the Supplemental Information available with this article online), we observed 100% diabetes induction only in response to suprathreshold (i.e., above the threshold for negative selection) antigens (Figure 1A). Diabetes induction was not simply due to the activation of high numbers of antigen-specific T cells because as few as 1.5×10^5 OT-I T cells were sufficient to induce diabetes after administration of the above-threshold peptide Q4R7 (Figure S1B). Similar to its effect in fetal thymic organ culture (Daniels et al., 2006), the T4 peptide variant exhibited threshold properties and induced diabetes in 25% of mice (Figure 1A). The below-threshold peptides Q4H7 and V4 were unable to induce diabetes in this model. These findings indicate a striking threshold effect in light of the fact that the OT-I TCR's affinity for Q4R7-K^b is only marginally higher (<2-fold) than its affinity for Q4H7-K^b (Figure 1A) (Daniels et al., 2006).

Because antigen dose and length of presentation can affect the efficiency of $CD8^+$ T cell activation, we attempted to further boost the initial priming of transferred OT-I T cells. However, repeated immunization with the below-threshold peptide Q4H7 was unable to sufficiently immunize OT-I T cells to generate autoimmune diabetes (data not shown). Mice receiving a 10-fold higher dose of Q4H7 (500 μg) exhibited a transient increase in urine glucose, which recovered to baseline within 2 weeks. This finding suggests that activation with subthreshold antigen was unable to promote sustained effector responses (Figure S1C). Although diabetes development in RIP-OVA mice can occur independently of help from CD4 T cells, it has been reported that fewer antigen-specific $CD8^+$ T cells are required to induce disease when help from CD4 T cells is abundantly available (Hernández et al., 2002; Kurts et al., 1997). To determine whether $CD4^+$ T cell activation would enable subthreshold ligands to induce diabetes, we coinjected RIP-OVA mice with 1×10^6 B6.C-H-2-bm12 B cells, which are allogeneic for host $CD4^+$ T cells (McKenzie et al., 1979). Provision of robust $CD4^+$ T cell helper responses, however, was inefficient in converting subthreshold peptides into disease-promoting ligands (Figure 1B). In contrast, the presence of $CD4^+$ T cells increased disease incidence in T4 immunized mice (25% without versus 80% with $CD4^+$ T cell help), which highlights the threshold properties of this peptide.

To eliminate potential effects from T cell competition and to more closely mimic an endogenous T cell response, we transferred 3×10^4 OT-I T cells into RIP-OVA recipients and then induced infection with recombinant *Listeria monocytogenes* expressing either Q4R7 or Q4H7 (Lm-Q4R7 or Lm-Q4H7) (Zehn et al., 2009). Despite the presence of a highly inflammatory bacterial infection, presentation of subthreshold Q4H7 was inefficient in inducing diabetes (2/14 versus 8/8 for the above-threshold Q4R7, Figure 1C). Taken together, these data indicate that the sharp affinity threshold characteristic of central tolerance (Daniels et al., 2006; Naeher et al., 2007) is utilized by peripheral $CD8^+$ T cells with respect to the generation of peripheral-tissue pathology.

TCR Affinity Dictates the Magnitude of Effector T Cell Activation

Because Lm-Q4H7 infection was previously shown to induce T cell expansion and effector responses (Zehn et al., 2009), we next examined the ability of OVA variant peptides to activate OT-I T cells in vivo. Q4R7 peptide administration induced rapid and high surface expression of CD25, CD69, LFA-1 (CD11a), and CD8 and resulted in a vigorous proliferative response (Figures 2A–2C). In contrast, Q4H7 immunization led to much

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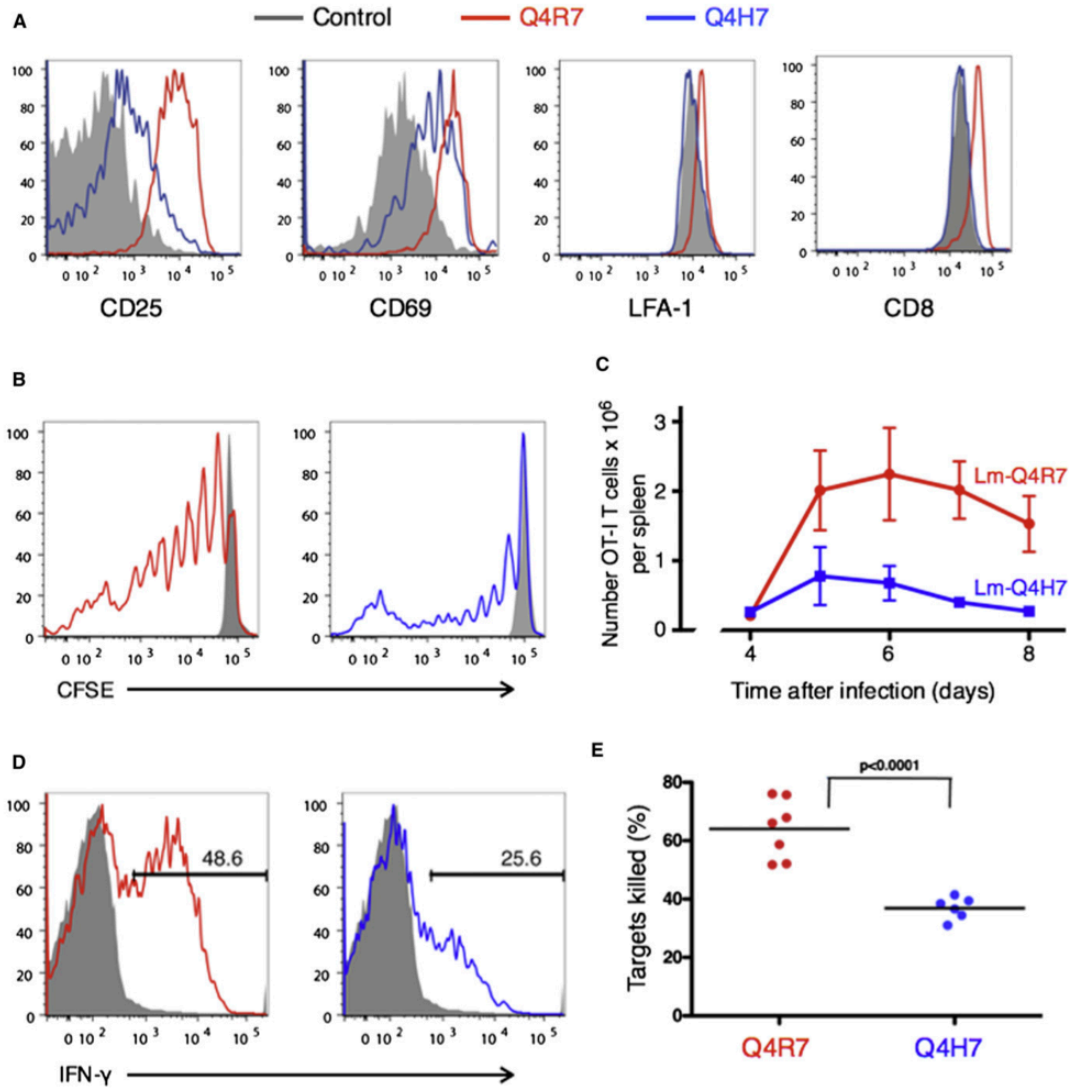

Figure 2. Characterization of In Vivo OT-I T Cell Responses after Immunization with Supratheshold or Subthreshold Peptide Ligands

RIP-OVA mice were injected with Rag-2-deficient OT-I cells, and immunization with peptide and LPS followed.

(A) Surface expression of CD25, CD69, LFA-1 (CD11a), and CD8 on lymph node OT-I T cells after 8 hr (n = 4 per peptide).

(B) CFSE dilution profiles of lymph node OT-I T cells 3 days after peptide immunization (n = 3 per peptide). The numbers of recovered OT-I donor cells from spleen and lymph nodes were equal to 2.2 ± 0.3 × 10^6 and 0.7 ± 0.1 × 10^6 for Q4R7 and Q4H7 immunized mice, respectively.

(C) Total numbers of splenic OT-I T cells in mice adoptively transferred with 3 × 10^4 OT-Is and 1 day later infected with Lm-Q4R7 or Lm-Q4H7 (two experiments, five mice per peptide).

(D) IFN-γ production by OT-I T cells restimulated for 5 hr with OVA peptide in vitro (n = 6 per peptide).

(E) CTL activity from OT-I T cells. OVA ($CFSE^{hi}$) and VSV ($CFSE^{lo}$) peptide-pulsed splenocytes were mixed at a 1:1 ratio and injected into RIP-OVA mice 3 days after peptide immunization. Target cell lysis was analyzed from splenocytes 5 hr after cell transfer. CTL activity from unimmunized mice was <10% (n = 7, Q4R7; n = 6 Q4H7). Representative data are shown for n ≥ 2 separate experiments. Error bars denote standard error of the mean (SEM).

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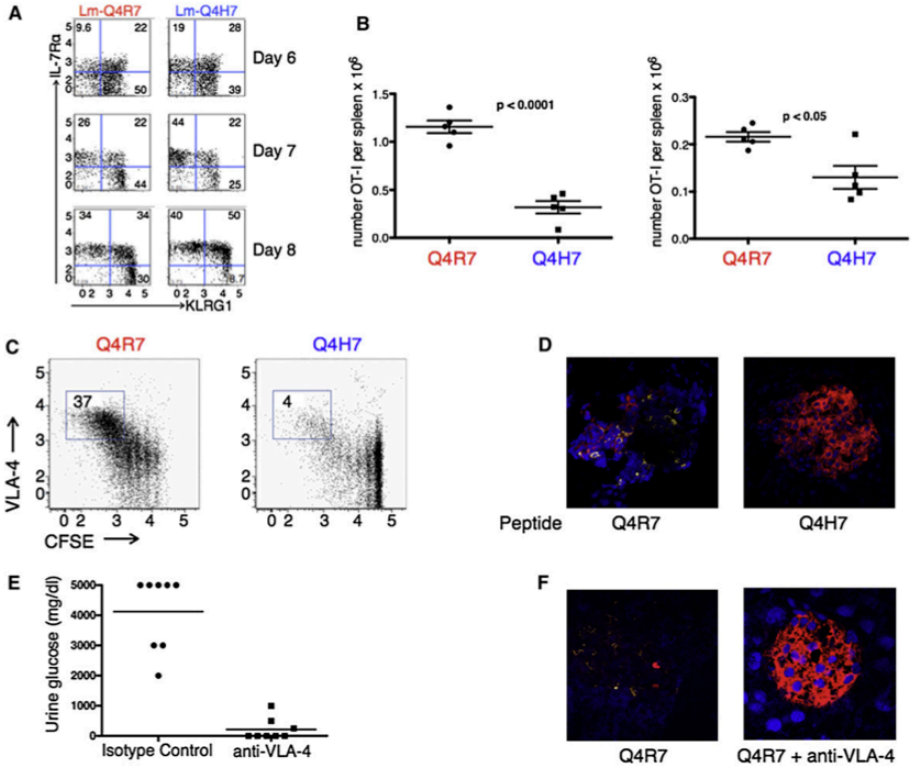

Figure 3. T Cell Differentiation and Tissue Infiltration after Immunization with Suprathreshold or Subthreshold Peptide Ligands

(A and B) OT-I T cells were adoptively transferred into C57BL/6 mice, and infection with Lm-Q4R7 or Lm-Q4H7 followed. (A) Representative flow-cytometry plots of SLEC and MPEC cells at days 6, 7, and 8 after infection. Numbers on the axes represent the log10 of fluorescence.

(B) Total splenic OT-I T cell numbers of SLEC and MPEC cells at day 6 after infection. Representative data are from two separate experiments; n = 5 mice per peptide; statistical analysis was performed with an unpaired two-tailed Student's t test. Error bars denote SEM.

(C–F) T cell infiltration and diabetes induction mediated by VLA-4 upregulation on proliferating T cells. CFSE-labeled congenic OT-I T cells (5×10^6) were injected into RIP-OVA recipients, and peptide immunization and LPS followed. (C) Surface expression of VLA-4 and CFSE dye dilution on LN T cells was determined at day 3. Numbers on the axes represent the log10 of fluorescence. Representative data are from two separate experiments; n = 4 mice per peptide. (D) Representative immunohistochemistry for CD8 (yellow), insulin (red), and DAPI (blue) on pancreatic sections taken 3 days after immunization; images are magnified 40× (n = 3). (E and F) Mice were treated with anti-VLA-4 (500 μg, clone PS2) or isotype control (500 μg, IgG2b) at days 0 and 2. (E) Urine glucose (n = 8) and (F) immunohistochemistry (n = 3) were examined at day 4.

lower expression of these activation markers and stimulated less proliferation. Despite their reduced proliferation, Q4H7-stimulated OT-I T cells were able to produce interferon-γ (IFN-γ) in response to re-stimulation with OVA peptide in vitro and exhibited efficient CTL function in vivo (Figures 2D and 2E) consistent with the observations of Zehn, et al. (Zehn et al., 2009). Importantly, the magnitude of Q4R7- or Q4H7-induced effector T cell responses directly correlates with the ability of these peptides to induce T cell expansion.

T Cell Differentiation and Tissue Infiltration

High expression of CD25 promotes the development of IL-7Rα^{lo} KLRG1^{hi} short-lived effector cells (SLECs) and drives effector T cell proliferation later in the immune response (Obar et al., 2010). Indeed, *Il2ra*$^{-/-}$ CD8$^+$ T cells exhibit impaired differentiation of SLECs despite initial robust expansion. To investigate the effect of TCR affinity on SLEC differentiation in vivo, we examined the expression of IL-7Rα and killer cell lectin-like receptor G1 (KLRG1) by flow cytometry. At the peak of T cell proliferation after Listeria infection (day 6), the frequency of SLECs (IL-7Rα^{lo} KLRG1^{hi}) was higher in T cells activated by the suprathreshold peptide Q4R7 than in T cells activated by the below-threshold antigen Q4H7 (Figure 3A). This increased frequency of SLECs in Lm-Q4R7-infected mice became even more prominent at days 7 and 8 after infection, when T cell contraction has begun to occur (Figure 3A). In contrast, the frequency of memory-precursor (MP) effector cells (IL-7Rα^{hi}KLRG1^{lo}) was decreased in mice infected with Lm-Q4R7 as compared to mice infected with Lm-Q4H7 (Figure 3A). These shifts in frequency primarily reflected an increase in SLEC numbers as a consequence of

stimulation with suprathreshold Q4R7 antigen, although MPEC numbers were somewhat increased as well (Figure 3B).

The suprathreshold peptide Q4R7 also induced the upregulation of VLA-4 (also called CD49d or α4 integrin), which was maximally expressed on $CD8^+$ T cells that had undergone more than five divisions (Figure 3C). Q4H7 activated T cells did not undergo as vigorous proliferation, although the few T cells that had progressed beyond five divisions also showed increased VLA-4 expression. Interaction of VLA-4 with VCAM-1 expressed on pancreatic endothelium has been shown to regulate T cell infiltration into the pancreas (Hänninen et al., 2007). Indeed, immunization with the suprathreshold peptide Q4R7 resulted in significant islet infiltration by $CD8^+$ T cells, whereas no significant infiltration by subthreshold, Q4H7-activated T cells was observed (Figure 3D). Q4H7 induced minor infiltration late in the response at day 6, but this did not lead to any β cell loss (data not shown). Previous studies have shown that treatment with anti-VLA-4 can inhibit the homing and infiltration of antigen-specific T cells in several autoimmune models (Burkly et al., 1994; Yednock et al., 1992). To determine whether Q4R7-mediated VLA-4 upregulation was responsible for islet infiltration in RIP-OVA mice, we treated immunized mice with mAb to VLA-4 at the onset of Q4R7 priming. All control mice developed diabetes and exhibited substantial islet infiltration by $CD8^+$ T cells, whereas anti-VLA-4-treated mice remained healthy (Figures 3E and 3F). These data indicate that, despite the ability of Q4H7 to induce effector T cell responses, the scarcity of T cells expressing VLA-4 reduces islet infiltration and is therefore a limiting factor in disease development.

TCR Affinity Regulates T Cell-APC Conjugation

We wondered how the marginal affinity difference (~1.5 fold) between the Q4R7 and the Q4H7 APLs can result in such different phenotypic and functional outcomes. One of the important factors governing T cell activation is the ability of T cells to form long-lasting conjugates with APCs (Iezzi et al., 1998). Splenocyte APCs pulsed with the suprathreshold Q4R7 formed 10-fold more conjugates with OT-I T cells than did Q4H7-pulsed splenocytes (Figure 4A), which is consistent with Q4R7's ability to promote enhanced T cell proliferation. Higher concentrations of Q4H7 did not lead to increased conjugate formation (Figure 4B) or maximal expression of CD25 (Figure 4C) on OT-I T cells, even though T cell proliferation was enhanced. Despite their inability to form long-lasting interactions with APCs, OT-I T cells stimulated with subthreshold ligands are able to enter the cell cycle. They do not, however, maintain a high level of CD25 expression, which probably limits their capacity for sustained expansion (van Stipdonk et al., 2003). In contrast, above-threshold ligands such as Q4R7 induce stable conjugates, high CD25 expression, and sustained proliferation (Figures 2B, 2C, and 4A–4C).

The formation of T cell-APC conjugates is critically dependent on the TCR's ability to activate LFA-1 (Scholer et al., 2008). To examine the contribution of TCR affinity to LFA-1 activation, we incubated OT-I T cells with K^b-Q4R7 or K^b-Q4H7 tetramers and measured T cell adhesion to plate-bound purified mouse ICAM-1, the ligand for activated LFA-1. K^b-Q4R7 stimulation generated 3-fold more ICAM-1-adherent OT-I cells than stimulation with subthreshold K^b-Q4H7 (Figure 4D). This increase was mediated by LFA-1 because adhesion could be inhibited with an antibody blocking LFA-1 function. Given the importance of LFA-1-ICAM interactions for generating sustained T cell-APC contacts (Scholer et al., 2008), we next examined the effect of antigen affinity on the duration of conjugation by using time-lapse videomicroscopic imaging to track individual DC-T cell pairs. As expected, OT-I T cells formed more conjugates with Q4R7-loaded DCs than with Q4H7-loaded DCs (11.7 ± 2.5% for Q4R7 versus 2.3 ± 0.7% for Q4H7, $p < 0.005$). In addition, OT-I-Q4R7-loaded APC conjugates were of much longer duration than OT-I -Q4H7 loaded APC conjugates (Figure 4E and Movie S1). This difference is conspicuous given the minimal difference between K^b-Q4R7 and K^b-Q4H7 in affinity for the OT-I receptor.

TCR Affinity Regulates Asymmetric Cell Division

Sustained synapses between T cells and APCs were also shown to be required for the asymmetric division of $CD8^+$ T cells responding to bacterial infection (Chang et al., 2011; Chang et al., 2007). After T cell activation, CD8 and LFA-1 redistribute to the immune synapse, and the resulting polarization sets the stage for asymmetric division, where one daughter cell (proximal [P] to the synapse) recruits greater amounts of CD8 and LFA-1 than the other (distal [D] to the synapse) (Chang et al., 2007; Dustin and Chan, 2000; Oliaro et al., 2010). To determine whether stimulation with the suprathreshold Q4R7 resulted in more asymmetric division than that with Q4H7, we transferred CFSE-labeled naive OT-I T cells into congenic recipients and followed this by immunization with peptide and LPS. After 24 to 36 hr, undivided T cells were sorted and either used directly for confocal analysis (Figure 5A) or cultured in vitro for several hours (Figure 5B) (Chang et al., 2007). A polarized distribution of CD8 was observed in Q4R7-activated mitotic T cells, which could be identified by the presence of two oppositely facing microtubule organizing centers (MTOCs). Most CD8 colocalized with only one of the MTOCs (Figure 5A, rows 1 and 2). In addition, Q4R7-generated conjoined daughter cells maintained this asymmetry; one daughter cell inherited the majority of CD8 and LFA-1 (Figures 5B and 5C). Analysis of evolutionarily conserved polarity proteins also revealed an asymmetric distribution, in that Scribble segregated to the putative proximal daughter and PKCς segregated to the distal daughter. In addition, Numb, which is an inhibitor of Notch signaling and has a conserved role in asymmetric cell division (Betschinger and Knoblich, 2004), was preferentially localized in proximal daughter cells (Figures 5B and 5C). These findings are in agreement with the previously reported asymmetric localization of these proteins after T cell activation (Chang et al., 2011; Chang et al., 2007).

In contrast, Q4H7-activated T cells exhibited more-uniform CD8 staining prior to division (Figure 5A, rows 3 and 4), and the majority of conjoined daughter cells contained equivalent levels of synaptic proteins (CD8, LFA-1), polarity proteins (Scribble, PKCζ), and Numb (Figures 5B and 5C). A similar symmetric pattern of staining has been reported for T cells undergoing homeostatic proliferation in $Rag^{-/-}$ mice which is driven by low affinity self antigens, i.e., those that induce positive selection in the thymus (Chang et al., 2007; Ernst et al., 1999; Goldrath and Bevan, 1999a). Although Q4H7 was originally described as a strong positive selector in OT-I fetal thymic organ culture (Daniels et al., 2006), it is capable of inducing peripheral

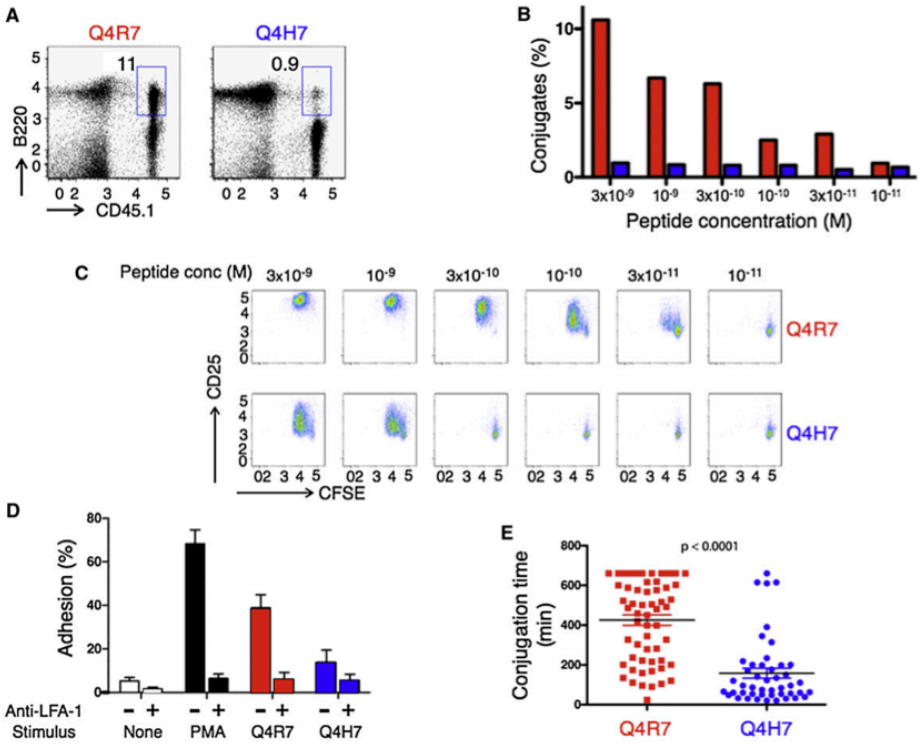

Figure 4. Conjugate Formation between OT-I T Cells and APCs Pulsed with Suprathreshold or Subthreshold Peptide Ligands

(A) APCs (C57BL/6 splenocytes) were incubated with 1×10^{-8} M peptide and LPS and mixed with congenic (CD45.1) OT-I lymphocytes. Conjugate formation was determined after antibody staining and flow cytometry. Numbers on the axes represent the log10 of fluorescence. Representative data are from four independent experiments.

(B) Splenocytes were pulsed with the indicated concentration of peptide and cultured together with OT-I T cells.

(C) Proliferation (CFSE dye dilution) and CD25 expression was assessed after 48 hr. Numbers on the axes represent the log10 of fluorescence.

(D) Adhesion of OT-I T cells to mouse ICAM-1 was determined after no stimulation or after incubation with tetramer (K^{b-}Q4R7 or K^{b-}Q4H7) or PMA (Mueller et al., 2004). Results are expressed as the mean percentage of adherent cells from triplicate wells ± SD. Representative data are from three independent experiments.

(E) Bone-marrow-derived dendritic cells were labeled with PKH-26 and incubated with 1×10^{-8} M peptide and LPS. After 4 hr, CMAC-blue-labeled OT-I T cells were added, and individual T cell-DC conjugates were tracked over a 12 hr period by time-lapse videomicroscopy. The horizontal bars indicate the average conjugation time ± SEM. Statistical analysis was performed by an unpaired two-tailed Student's t test. (see also Movie S1 and Movie S2).

T cell proliferation (Figure 2B). However, this proliferation is more likely to result in symmetric divisions (Figures 5B and 5C) that do not fully promote effector differentiation (Figure 3).

The dependence of asymmetric division on LFA-1-ICAM interactions (Chang et al., 2007) and the inability of subthreshold ligands to induce strong LFA-1 activation, efficient conjugation, or asymmetric division (Figures 4 and 5) indicate a role for long-lasting T cell-APC contacts in regulating T cell polarity. To investigate whether prolonged T cell-APC contacts induced by high-affinity peptides are a factor in initiating asymmetric division, we examined the subcellular localization of PKCζ in dividing OT-I T cells activated by Q4R7 pulsed DCs. After 4 or 24 hr of coculture, T cell-APC conjugates were mechanically disrupted, and T cells were further cultured with LPS-activated DCs in the absence of peptide for an additional 44 or 24 hr, respectively. In-vitro-activated OT-I T cells undergoing prolonged coculture with Q4R7-loaded APCs exhibited asymmetric distribution of PKCζ (16/19 asymmetric cells, Figure 5D). Asymmetric division correlated with higher expression of CD25 at 72 hr (Figure S2). In contrast, T cells cocultured for only 4 hr with Q4R7-pulsed DCs primarily exhibited symmetric distribution of PKCζ and correspondingly lower levels of CD25 (4/16 asymmetric cells, Figure 5D; see also Figure S2). Taken together, these data reveal a requirement for sustained contact between T cells and APCs for the establishment of T cell polarity and asymmetric division.

Asymmetric Division Promotes SLEC Differentiation and Tissue Infiltration

Asymmetric T cell division results in the generation of putative proximal and distal daughter cells with high and low amounts of CD8, respectively. The CD8 coreceptor plays a role in

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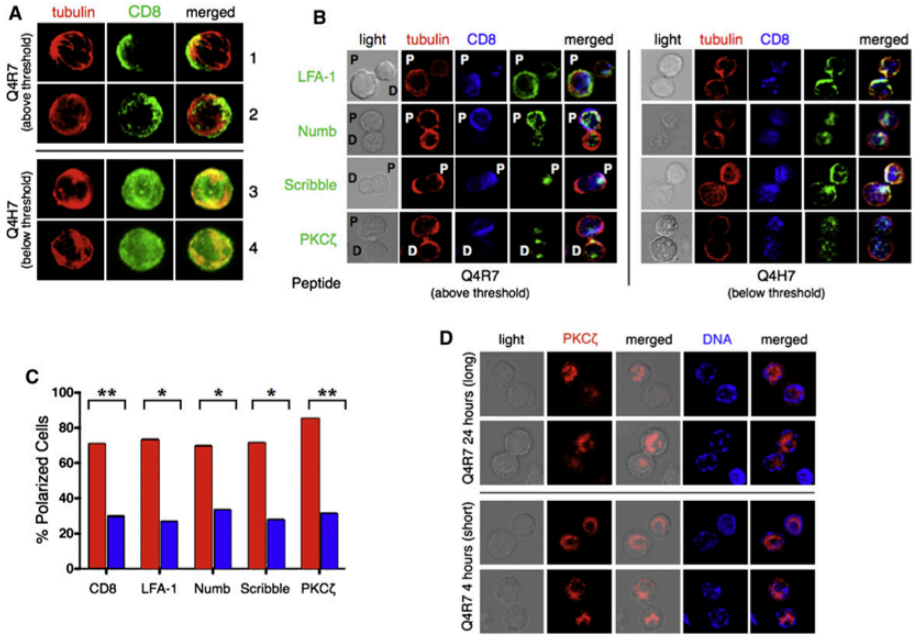

Figure 5. Assessment of Asymmetric Cell Division in OT-1 T Cells Responding to Suprathreshold or Subthreshold Peptide Ligands

CFSE-labeled OT-I T cells were transferred into C57BL/6 mice, and immunization with LPS and either Q4R7 or Q4H7 followed. After 24 to 36 hr, undivided (brightest CFSE peak) OT-I T cells were sorted and analyzed by confocal microscopy for (A) β-tubulin and CD8 localization on single T cells or (B) β-tubulin, CD8, LFA-1, Scribble, Numb, and PKCζ on conjoined daughter cells (Chang et al., 2007; Oliaro et al., 2010; Suzuki and Ohno, 2006). Proximal (defined as the cell with a majority of CD8 expression) and distal daughters are marked with "P" and "D," respectively. (C) Quantification of asymmetric protein distribution in conjoined daughter T cells shown in (B). The number of conjoined daughter cells analyzed for each stain is indicated in parentheses as follows (nr Q4R7, nr Q4H7): CD8 (72, 67), LFA (15, 15), Numb (23, 18), Scribble (14, 18), and PKCζ (20, 16). *$p \leq 0.02$; **$p \leq .001$. Statistical analysis was performed by a two-tailed Student's t test. (D) CFSE-labeled T-1 T cells were cultured for 4 or 20 hr with Q4R7-pulsed DCs and LPS. After mechanical disruption of conjugates, T cells were purified on a magnetic column and recultured with unloaded, LPS-treated DCs for an additional 24–44 hr. Mitotic T cells were analyzed by confocal microscopy for distribution of PKCζ and CFSE (n = 19 cells, Q4R7, n = 16 cells, Q4H7) (see also Figure S2).

stabilizing TCR-pMHC interactions, and CD8 binding is required for efficient conjugation between OT-I T cells and antigen-pulsed APCs (Jiang et al., 2011; Potter et al., 2001). To investigate whether differential expression of CD8 after asymmetric division has an effect on the subsequent binding of T cells to APCs, we sorted proximal ($CD8^{hi}$) and distal ($CD8^{lo}$) daughter cells from mice primed in vivo (Figures S3A, S3B, and S3C) and cultured the sorted daughter cells with peptide-pulsed DCs. Compared to distal daughters, proximal daughters formed twice as many conjugates with 10^{-8} M peptide-pulsed APCs, a difference that increased to 6-fold at lower (10^{-10} M) antigen concentrations (Figure 6A). This suggests that proximal daughters might have a competitive advantage over distal daughters when antigen becomes limiting or when T cell competition increases, as might occur during the later expansion phase of an immune response. To examine the proliferation and phenotype of proximal and distal daughter T cells in vivo, we injected sorted proximal and distal daughter cells separately into Lm-Q4R7-infected mice that had been infected 1 day or 3 days previously. Priming of $CD8^+$ T cells in response to *Listeria* has been shown to be most efficient one day after bacterial inoculation (Pamer, 2004). Donor T cells were analyzed at days 4 and 6 after transfer. In mice infected with Lm-Q4R7 for 1 day before T cell transfer, proximal daughters exhibited a ~2.5-fold increased accumulation compared to that of distal daughters after 6 days (Figure 6B). Accumulation in proximal daughters was further increased to approximately 8-fold more than that of distal daughters in mice that had been infected with Lm-Q4R7 for 3 days prior to T cell transfer (Figure 6B). Thus, the increased accumulation of proximal daughter T cells in mice that had been infected longer (3 days) prior to T cell transfer suggests that proximal daughters continue to expand even as antigen concentrations are decreasing from the time of T cell transfer (3 days after infection) to the end point of the experiment 6 days later. In this sense, they exhibit an increased competitive fitness over distal daughters. To examine potential alterations in T cell differentiation, we identified SLEC and MPEC populations by expression of IL-7Rα and KLRG1. Six days after transfer, the ratio of SLECs to MPECs was much higher in mice that had received proximal donor T cells than in mice receiving distal donor T cells (Figures 6C and 6D). Interestingly, T cells derived from a distal daughter also exhibited a large proportion of cells expressing both KLRG1 and IL-7Rα,

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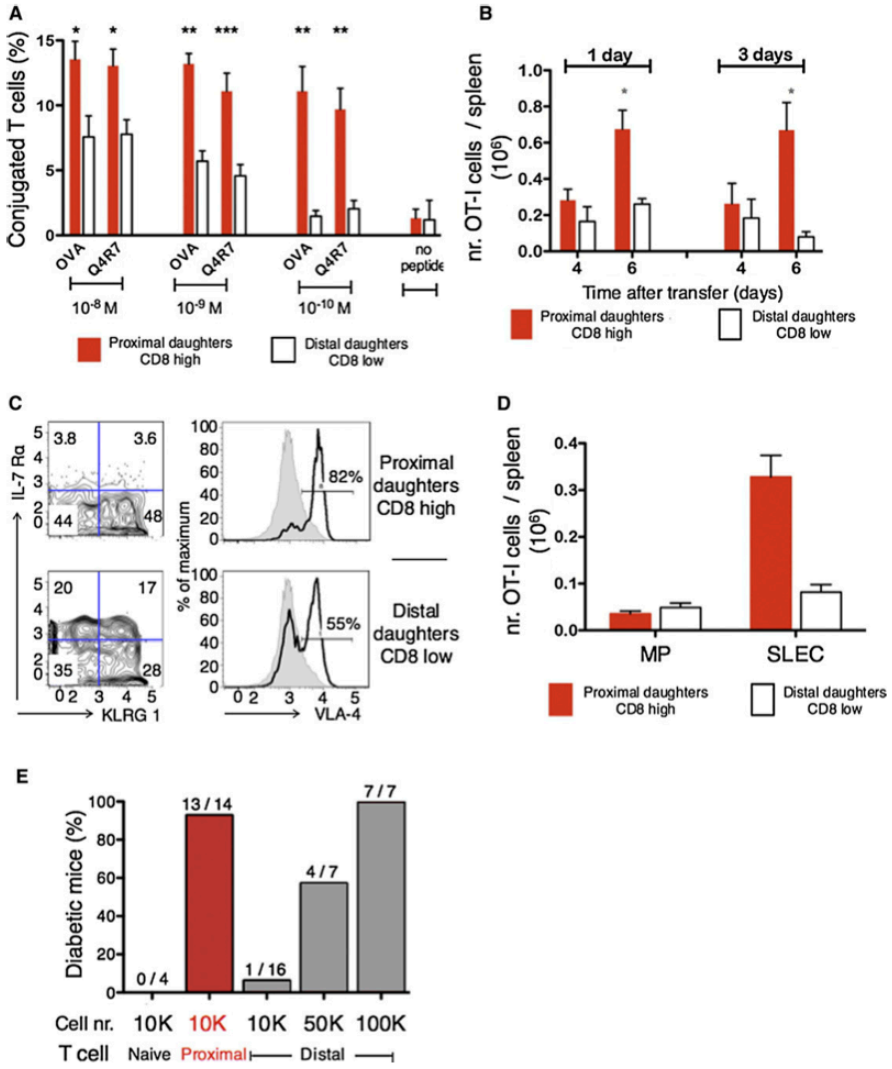

Figure 6. Phenotypic and Functional Characterization of CD8^{hi} and CD8^{lo} Daughter T Cells

CFSE-labeled OT-I T cells were injected into congenic mice, and immunization with peptide and LPS followed. After 24 hr (Q4R7), once-divided T cells (2nd CFSE peak), were sorted into CD8^{hi} (proximal daughter) and CD8^{lo} (distal-daughter) populations by FACS.

(A) Sorted T cells were cocultured with antigen-pulsed DCs, and conjugation was assessed by flow cytometry after 20 min incubation (n = 3 experiments). *p < 0.005 by an unpaired two-tailed Student's t test. Results are expressed as the mean percentage of conjugated cells ± SD.

(B–D) Sorted T cells were adoptively transferred into congenic mice that had been infected with Lm-Q4R7 1 or 3 days previously (three experiments, n = 3 mice per group). *p < 0.005. (B) Total splenic OT-I T cell numbers at days 4 and 6 after T cell transfer. (C) Representative FACS plots of KLRG1, IL-7Rα, and VLA-4 6 days after T cell transfer into mice that had been infected 72 hr prior to transfer. Numbers on the axes represent the log10 of fluorescence. (D) Total splenic OT-I T cell numbers of SLEC and MPEC cells at day 6 after infection (three experiments, n = 3 mice per group). *p < 0.001 (unpaired two-tailed Student's t test). Results in (B) and (D) are expressed as the mean number of cells from triplicate mice ± SD.

(E) Sorted proximal or distal daughters were injected into RIP-OVA mice that had been infected with Lm-Q4R7 1 day previously. Diabetes development was assessed as in Figure 1 (see also Figures S3D and S3H).

although the function of these cells is not known (Figure 6C). In addition, a higher proportion of the progeny of proximal daughters expanding in Lm-Q4R7-infected mice had increased expression of VLA-4 (Figure 6C).

To determine whether Q4R7-induced asymmetric cell division led to daughter cells with different potentials to induce tissue pathology, we sorted proximal ($CD8^{hi}$) and distal ($CD8^{lo}$) daughters and adoptively transferred them into RIP-OVA mice that had been infected 1 day previously with Lm-Q4R7. Although 10^4 naive OT-I T cells are less than the numerical threshold required to induce diabetes, the same number of proximal daughter cells induced diabetes in 90% (13 of 14) of Lm-Q4R7-infected mice (Figure 6E). In contrast, 10^4 distal daughter cells did not cause disease in similarly infected mice (1 of 16). It should be noted that distal daughters were able to cause disease in Lm-Q4R7-infected mice, but 5- to 10-fold higher numbers of transferred distal daughters were required to generate a similar frequency of diabetes (Figure 6E). Similarly, smaller numbers of distal daughters were sufficient to induce disease in mice infected with Lm-OVA (Figure S3D), which induced stronger and more sustained proliferation (Figure S3E).

Proximal daughters responding to Q4R7 in vitro exhibited both more proliferation and higher expression of CD25 than did distal daughters (Figure S3F). Given the importance of IL-2 signaling in SLEC differentiation, it's possible that the efficiency in SLEC differentiation shown by proximal daughters responding to Q4R7 stimulation in vivo is a result of higher CD25 expression, which in turn influences the extent of T cell proliferation. Proximal daughters responding to OVA peptide in vitro also exhibited more proliferation than distal daughters; however, CD25 expression, SLEC/MPEC ratios, and VLA-4 expression were similar in both populations (Figures S3F and S3G). It's conceivable that distal daughters can re-enter the proliferating T cell pool and differentiate into SLECs if they encounter an even higher-affinity antigen.

Taken together, these data highlight the importance of T cell expansion in causing diabetes. Although proximal daughters have a clear advantage over distal daughters in their ability to expand, transferring higher numbers of distal daughters or exposing distal daughters to the highest-affinity antigen, Lm-OVA, which elicits stronger and more sustained proliferation (Zehn et al., 2009), are sufficient to induce diabetes.

In summary, these data show that suprathreshold antigens lead to long T cell-APC contacts, asymmetric division, and the generation of polarized proximal daughter T cells. $CD8^{hi}$ proximal daughters are significantly better able to make conjugates with antigen-bearing APCs, expand, and differentiate into SLECs expressing VLA-4, a molecule required for T cell infiltration into peripheral tissues. Consistent with these observations is our finding that OT-I T cells primed with suprathreshold Q4R7 in ICAM-deficient ($Icam1^{-/-}$) hosts, which are unable to support asymmetric division (Chang et al., 2007), were less efficient at inducing disease after transfer into Lm-Q4R7-infected RIP-OVA mice (Figure S3H).

DISCUSSION

The experiments presented here show that an antigen affinity threshold determines the differentiated state of a responding T cell. Previous work revealed that the boundary between positive and negative selection occurs across a very sharp TCR affinity threshold (Daniels et al., 2006), but whether this affinity threshold is similarly used by peripheral $CD8^+$ T lymphocytes for the maintenance of peripheral tolerance was not previously known. Our results demonstrate that only antigens above the negative selection threshold are able to fully promote the development of effector T cells capable of infiltrating the pancreas and efficiently causing β cell destruction. It's striking that the disease-promoting ligand K^b-Q4R7 has only a marginally higher (~1.5 fold) affinity for OT-I cells than does K^b-Q4H7 (Daniels et al., 2006), which is inefficient at causing diabetes in RIP-OVA mice. This emphasizes the importance of this affinity threshold for peripheral $CD8^+$ T cell responses. That Q4R7 is an efficient negative selector (Daniels et al., 2006) underscores the importance of central tolerance in preventing highly self-reactive thymocytes from entering the peripheral repertoire.

Our data also highlight a requirement for an above-threshold antigen affinity to generate sustained T cell-APC contacts in order to maintain primary T cell expansion. ICAM-LFA-1 signals are essential for long-lasting T cell-DC conjugates (Scholer et al., 2008), and here we show that T cell activation with above-threshold ligands induces sustained T cell-APC contacts mediated by ICAM-LFA-1 interactions. Although we have not examined the morphology of OT-I -APC synapses, a recent study (Schubert et al., 2012) demonstrates that low-affinity, autoreactive $CD4^+$ helper T cells exhibit atypical immune synapses. It would be interesting to determine whether the synapse is organized differently when a $CD8^+$ T cell recognizes a subthreshold antigen. Although ICAM-1 appears to be dispensable for the initial priming and differentiation of effector T cells, ICAM-1 deficiency results in decreased accumulation of effector T cells at later time points (Scholer et al., 2008). In addition, despite normal acquisition of effector function, LFA-1-deficient T cells were recently shown to have a specific defect in SLEC differentiation (Beinke et al., 2010). Interestingly, we observed normal cytokine production and cytotoxicity by T cells activated with below-threshold ligands. Indeed, subthreshold antigen stimulation has previously been shown to induce T cell proliferation and effector function, but the magnitude of these responses is reduced, and T cell contraction occurs earlier (Zehn et al., 2009). The earlier contraction observed in response to subthreshold activation most likely prevents the full accumulation of SLECs that mediate pancreatic β cell destruction. In this context, we observed decreased numbers of SLEC effector T cells in response to subthreshold TCR stimulation. Of note, although $Il2ra^{-/-}$ $CD8^+$ T cells exhibit general defects in expansion and survival after Listeria infection, the most significant decrease is observed in the SLEC subset, indicating the dependence of this population on IL-2 signaling. (Obar et al., 2010).

Importantly, our data reveal asymmetric division as an important mechanistic link between increased signal strength and T cell differentiation (Dustin and Chan, 2000). Previous work demonstrated that short stimulation leads to decreased CD25 expression and abortive clonal expansion while long stimulation promotes sustained proliferation and CD25 expression (Gett et al., 2003; van Stipdonk et al., 2003). Our data expand on these observations by showing that prematurely disrupting T cell-APC conjugates prevents asymmetric division and a concomitant increase in CD25 expression. In agreement with this,

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T cells primed with suprathreshold ligand in the absence of ICAM-LFA-1 signals, which are essential for long-lasting DC interactions as well as asymmetric division, are less efficient at causing diabetes when transferred into RIP-OVA mice (Chang et al., 2007; Scholer et al., 2008).

Asymmetric division results in the generation of $CD8^{hi}$ daughter cells with greater capacity than $CD8^{lo}$ daughter cells to bind antigen-loaded APCs. Higher expression of LFA-1 on $CD8^{hi}$ proximal daughters is also likely to be a factor in their enhanced conjugation with APCs (Chang et al., 2007). In vivo, these $CD8^{hi}$ daughter cells undergo enhanced proliferation, exhibit increased differentiation into SLECs and have a higher proportion of cells expressing VLA-4, which is required for tissue infiltration. Importantly, $CD8^{hi}$ proximal daughter cells are much more efficient at infiltrating peripheral tissues and inducing target cell destruction. The enhanced APC binding and differentiation of $CD8^{hi}$ proximal daughter cells reveals the importance of repeated T cell-APC encounters that occur after the onset of T cell division (Celli et al., 2005). In contrast, $CD8^{lo}$ distal daughter cells exhibited impaired accumulation of tissue-infiltrating SLECs. Interestingly, a large proportion of T cells derived from $CD8^{lo}$ distal daughters were double positive for both KLRG1 and IL-7Rα. A similar population of double-positive cells has been observed in SLP-76-deficient T cells (Smith-Garvin et al., 2010), suggesting that lower levels of T cell activation lead to incomplete or transient downregulation of IL-7Rα expression. Taken together, the data presented here suggest a scenario in which above-threshold antigens activate LFA-1 on responding T cells and lead to long-lasting conjugates and asymmetric cell division. This sequence generates $CD8^{hi}$ proximal daughter cells, which probably further upregulate CD25 expression, leading to the cells' sustained proliferation and differentiation into tissue-infiltrating SLECs.

A similar requirement for T cell polarity in SLEC differentiation was reported for both Pyk2-deficient and LFA-1 deficient T cells, which exhibit impaired SLEC differentiation after LCMV infection (Beinke et al., 2010). Interestingly, as antigen concentrations decrease, $CD8^{hi}$ proximal daughters maintain their ability to bind strongly to antigen-bearing DCs in vitro as well as to proliferate in vivo. This contrasts with $CD8^{lo}$ distal daughters, which exhibit decreased binding to antigen-bearing DCs and decreased proliferation at lower antigen levels. These findings raise the possibility that as T cell numbers and competition for antigen increases, only above-threshold ligands are able to coordinate the polar distribution of effector fate determinants, support continued T cell expansion, and induce the acquisition of surface molecules that allow infiltration of peripheral tissues. Of note, $CD8^{lo}$ distal daughter T cells were able induce disease when 5- to 10-fold higher numbers of cells were transferred. One possible explanation is a contamination of the sorted $CD8^{lo}$ distal daughter population with a small number of $CD8^{hi}$ proximal daughter cells capable of undergoing extensive proliferation and differentiation into disease-inducing effectors. Nevertheless, distal daughters can also cause disease when transferred into RIP-OVA mice infected with Lm-OVA, which induces more sustained T cell proliferation. $CD8^{lo}$ distal daughters might be better able to respond to OVA, given the OT-I receptor's higher affinity and lower CD8 dependence for binding K^b-OVA than for binding K^b-Q4R7 (Daniels et al., 2006).

Asymmetric division also results in the unequal partitioning of the transcription factor T-bet (Chang et al., 2011; Chang et al., 2007), which is known to repress IL-7Rα expression and drive SLEC differentiation (Intlekofer et al., 2007; Joshi et al., 2007). Importantly, T-bet-deficient $CD8^+$ T cells exhibit defective effector T cell accumulation and are unable to mediate diabetes in a RIP-LCMV system (Juedes et al., 2004). Thus, in addition to differences in CD8 and LFA-1 expression, asymmetric division most likely leads to differential expression of other fate-determining factors in proximal daughters, which also promote the differentiation of tissue-infiltrating SLECs.

One general question we have not yet resolved is whether above-threshold antigens principally drive extensive T cell proliferation, which subsequently leads to asymmetric division and differentiation. Alternatively, above-threshold antigens might establish T cell polarity and asymmetric division early on in the T cell response; this initial differentiating event might propel the T cell to undergo extensive division and concomitantly acquire a SLEC phenotype.

In summary, a TCR-pMHC affinity above the negative selection threshold (K_D = 6 μM) is required for efficient induction of asymmetric division, enhanced T cell proliferation, and differentiation into SLECs capable of tissue infiltration and target cell destruction. Antigens below this affinity threshold are much less efficient at inducing asymmetric T cell division, sustaining T cell proliferation, and inducing SLEC differentiation. Finally, these data raise the question of whether autoimmunity arises when failures in thymic selection allow a small number of high-affinity, self-reactive T cells to enter the peripheral repertoire or when there is chronic stimulation of below-threshold T cells. In this respect, it's interesting that Q4H7 induces asymmetry in some OT-I T cells, which argues for the latter possibility.

EXPERIMENTAL PROCEDURES

Mice

All animal work was done in accordance with the Federal and Cantonal laws of Switzerland. Wild-type C57BL/6, C57BL/6 *Rag2*$^{-/-}$, CD45.1 congenic mice and OT-I TCR transgenic mice recognizing OVA peptide 257-264/Kb were bred in our colony. RIP-OVA mice (Behrens et al., 2004) were obtained from Jackson Laboratories (Bar Harbor, ME).

Adoptive Transfers, Immunization, and Infection with *Listeria monocytogenes*

For diabetes induction, 5 × 10^6 OT-I $CD8^+$ T cells were adoptively transferred intravenously into RIP-OVA mice 1 day prior to immunization with 50 μg peptide and 25 μg LPS. Urine glucose was monitored daily. Alternatively, RIP-OVA mice received 3 × 10^4 OT-I T cells 1 day prior to infection with *L. monocytogenes* expressing Q4R7, Q4H7, or OVA (Zehn et al., 2009). The bacteria were grown to mid-log phase, and 5,000 colony-forming units (CFUs) were then injected intravenously into mice. For confocal microscopy analysis, 5 × 10^6 CFSE-labeled OT-I T cells were transferred into C57BL/6 mice, and immunization with peptide and LPS followed. After 24–48 hr, undivided T cells (brightest CFSE peak) were sorted from spleens and lymph node and cultured as previously described (Chang et al., 2007).

T Cell Proliferation, Cytokine Production, and Killing Assays

OT-I $CD8^+$ T cells were labeled with 5 μM CFSE and adoptively transferred into RIP-OVA mice (5 × 10^6 CFSE+ T cells/mouse). One day later, mice were immunized with 50 μg Q4R7 or Q4H7 and 25 μg LPS. At day 3 after immunization, peripheral lymph nodes (axillary, inguinal, and cervical) and spleens were harvested, counted, and pooled. Fluorescence-activated cell sorting (FACS) analysis of CFSE dye dilution was used for assessment of proliferation. For the

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detection of intracellular cytokines, splenocytes from day-3-immunized RIP-OVA mice were stimulated with OVA peptide in vitro in the presence of monensin for 4 hr at 37°C. For assessment of CTL function in vivo, splenic APCs from CD45.1 congenic mice were labeled with 5 μM ($CFSE^{hi}$) or 0.5 μM ($CFSE^{lo}$) CFSE. $CFSE^{hi}$ and $CFSE^{lo}$ cells were pulsed for 4 hr at 37°C with 2 μM OVA or VSV peptide, respectively. Cells were mixed at a 1:1 ratio, and 10^7 APCs were injected intravenously into recipients harboring activated OT-I T cells. Surviving APCs were determined after 5 hr by flow cytometry.

T Cell Adhesion Assay

Adhesion of 1×10^6 OT-I T cells to mouse ICAM-1 was determined after stimulation with 5 μg/ml tetramer (K^bQ4R7 or K^bQ4H7) or PMA (10 ng/ml) (Mueller et al., 2004). LFA-1 blocking was carried out with 10 μg/ml CD11a mAb. T cells were incubated at 37°C for 15 min on ICAM-1-coated plates and washed so that nonadherent cells would be removed; adherent cells were then eluted and counted by Trypan blue exclusion.

Confocal Microscopy

Cells were placed on poly-L-lysine-coated coverslips (BD PharMingen), fixed with 4% formaldehyde (Polysciences), permeabilized with 0.3% Triton X-100 (Sigma) and blocked with 0.25% fish skin gelatin (Sigma) and 1% normal mouse serum. Cells were stained with anti-β-tubulin (TUB 2.1, Sigma), anti-CD8, anti-CD11a (BD PharMingen), anti-Numb, anti-Scribble, or anti-PKCζ (Santa Cruz Biotechnology). Sections of 10–15 Z stacks were acquired with a LSM 780 confocal microscope (Zeiss). Z stacks were converted into two-dimensional images, and voxel volume was calculated with Imaris Software (Bitplane). Staining was considered asymmetric when one conjoined daughter cell expressed 1.75-fold higher staining than the corresponding conjoined twin daughter cell. For immunhistochemistry analysis, pancreas sections were stained with CD8 (BD PharMingen) and insulin antibodies (Dako).

SUPPLEMENTAL INFORMATION

Supplemental Information include three figures and two movies and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2012.06.021.

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7.2 Part II, Manuscript

Threshold affinity T cells provide the greatest risk for inducing experimental autoimmune diabetes

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T cell, Tolerance, TCR, Affinity, Autoimmunity

Abstract

T cell receptor affinity for self-peptide/MHC ligand has a well established role in thymic selection and in the initiation of autoimmune pathology. We were particularly interested to know how the affinity of TCR for p/MHC impacts the efficiency of negative selection, the ability to prime an autoimmune response and the ability of a target cell expressing a self-antigen to be lysed.

To address these questions, we generated three new transgenic lines expressing OVA-variant proteins of altered affinities for the OT-I TCR.

Adoptive transfer experiments suggest that below and threshold antigens poorly prime autoimmune responses, while above antigens are particularly efficient. Experiments with bone marrow chimeras allowed us to determine the effect of self-antigen affinity on the establishment of T cell tolerance. Our results demonstrate the efficiency of negative selection *in vivo* dramatically increases with above threshold self-antigens. However, mice expressing antigens just above the negative selection threshold exhibited the highest risk of developing experimental autoimmune diabetes upon immunization with the corresponding peptide self-antigen. These data argue that close to the affinity threshold for negative selection, sufficient numbers of self-reactive T cells escape deletion and create an increased risk for the development of autoimmunity.

Introduction

A protective and self-tolerant T cell repertoire is generated in the thymus (Palmer, 2003; Starr et al., 2003; Stritesky et al., 2012). The mechanism that promotes apoptosis of self-reactive T cells (negative selection) is dependent on the binding strength (affinity) between a T cell receptor (TCR) for self-peptide/MHC complex (pMHC). In this way central tolerance reduces the number of self-reactive T cells, minimalizing the risk for autoimmunity. (Goldrath and Bevan, 1999; Kisielow et al., 1988; von Boehmer et al., 1989).

Previous work defined the affinity threshold where negative selection is initiated (Daniels et al., 2006; Palmer, 2003). Experiments using three different MHC class I restricted TCR transgenic strains demonstrate that thymocytes undergo negative selection when binding a self-antigen with a KD ≤ 6 µM. MHC class I restricted thymocytes binding self-antigens with a lower affinity (KD > 6 µM) undergo positive selection (Daniels et al., 2006; Naeher et al., 2007). The fact that there is such a sharp affinity threshold distinguishing positive and negative selection indicates that the TCR reads antigen affinity with astonishing precision leading to a remarkable flexibility in signaling divergent thymocyte fates. Although central tolerance prevents most autoimmune T cells from entering the peripheral repertoire, negative selection is not a perfect process and a small number of high affinity, self-reactive T cells enter the periphery. (Taniguchi et al., 2012).

The transcription factor AIRE ensures that tissue-restricted antigens are also expressed in the thymus within resident medullary thymic epithelial cells (mTECs) (Metzger and Anderson, 2011). Aire-deficient thymic medullary epithelial cells have reduced levels of tissue restricted antigens (TRAs) on their surface, which in humans is known to induce multi-organ autoimmune disease (Anderson et al., 2002; Taniguchi et al., 2012). Since mTECs express a highly diverse set of genes including many tissue restricted antigens, the presentation of an individual antigen is limited on the mTEC surface (Anderson et al., 2002; Klein et al., 2009; Mathis and Benoist, 2009). In addition, individual self-antigens are expressed on only a small percentage of mTECs. Therefore, self-reactive T cells might stochastically escape negative selection, even in the presence of functional AIRE (Gotter et al., 2004).

A high frequency of self-reactive T cells in the peripheral repertoire directly correlates with the susceptibility to develop autoimmune disease. Studies with SJL mice showed that mice with high frequencies of myelin-specific T cells are more susceptible for the induction of EAE compared to mice with lower frequencies (Anderson et al., 2002). Studies where viral infection induced experimental autoimmune diabetes showed that a precursor frequency of ≥ 0.1% of self-reactive CD8 T cells is required to elicit disease (Sevilla, 2000). Ohashi et al. demonstrated that there is very little central deletion in P14 TCR x gp33 antigen double transgenic mice (Ohashi, 1991). This implies that there is limited thymic expression of the neo-self antigen in this model and likely explains the ease of diabetes induction in LCMV infected P14 TCR x gp33 antigen double transgenic mice (Ohashi, 1991). In gp33 single transgenic mice with a polyclonal T cell repertoire, cytotoxic T cells (CTL) generated during the immune response are of lower affinity compared to nontransgenic mice. This argues that in mice expressing the gp33 neo-self antigen, self-reactive T cells with highest affinity for the neo-antigen were removed from the T cell repertoire by central tolerance. Nevertheless, the remaining lower affinity T cells were capable of inducing diabetes upon LCMV infection. Similar findings have been reported for transgenic mice expressing the OT-I TCR β chain, which have an increased frequency of OVA reactive T cells (Zehn and Bevan, 2006).

Self-reactive T cells escaping thymic selection generally do not induce spontaneous autoimmunity. However, there are reports showing that priming of low affinity self-reactive T cells can occur when neo-self antigens are encoded by a viral or bacterial vector. Infection with these pathogens leads to a break in tolerance and subsequent autoimmune disease (Gronski et al., 2004; Ohashi, 1991; Oldstone et al., 1991; Zehn and Bevan, 2006). On the other hand, molecular mimicry proposes that a foreign-antigen can share structural or amino acid homology with a host derived antigen (Hudrisier et al., 2001). In most but not all cases of molecular mimicry the infectious foreign antigen has a higher affinity for the self-reactive T cell, compared to the T cell's affinity for its' self-antigen. This priming of low affinity autoimmune T cells is especially effective because the infection creates an inflammatory milieu.

How the affinity for activation of T cells relates to the negative selection threshold is not fully understood. Recently, King et al. found that the affinity

threshold established during central tolerance is maintained in periphery; only supra-threshold antigens (above the threshold for negative selection in the thymus) were able to generate fully differentiated effector T cells capable of initiating autoimmune diabetes in RIP-OVA mice (King et al., 2012). Strong interactions of TCR with above-threshold ligand drives T cells into asymmetric T cell division, generating proximal daughter cells which undergo sustained proliferation and differentiate into short-lived effector T cells. This study shows that T cell activation with above-threshold ligands induced sustained contact with antigen presenting cells (APCs). High-affinity interactions promote VLA-4 expression on effector T cells allowing their infiltration into the target tissue. In contrast, T cells with below threshold affinity TCRs are efficiently positively selected and are less likely to cause autoimmunity. Nevertheless, low affinity T cells can threaten the host because they can divide and acquire effector functions upon recognition of below threshold antigen in an infectious environment (Zehn et al., 2009b). In fact, Enouz et al. demonstrated that weak affinity T cells are indeed able to mediate autoimmunity after successive immunizations with recombinant *Listeria monocytogenes* (Lm) and Vesicular stomatitis virus (VSV) expressing self-antigen (Enouz et al., 2012).

Antigen affinity affects important parameters related to the development of autoimmunity: i) the efficiency of central tolerance, ii) the efficiency of T cell priming, and iii) the efficiency of destroying a target cell expressing the self-antigen.

In this study, we carried out experiments to examine these parameters separately and in combination. We show a striking threshold effect on central deletion, T cell priming and cytolysis of antigen expressing cells in the target tissue. These experiments demonstrate that thymocytes and their T cell descendants expressing TCRs close to the affinity threshold have the highest potential to induce an autoimmune disease.

Results

RIP-variant mice express variant OVA proteins in pancreatic β cells

In RIP-OVA mice, the transgenic rat insulin promoter (RIP) drives ovalbumin expression on β cells in the pancreas, the proximal tubular epithelial cells in the kidneys, and in medullary epithelial cells in the thymus (mTECs) (Kurts et al., 1997). To extend our knowledge of how self-antigen affinity affects the establishment of central tolerance, we generated several strains of RIP-variant transgenic mice, which express different OVA variants spanning the affinity threshold between positive and negative selection. Three novel transgenic mouse lines, expressing the membrane bound OVA variants, Q4H7 (RIP-mQ4H7, below threshold), T4 (RIP-mT4, threshold) or Q4R7 (RIP-mQ4R7, above threshold). As a source of OVA specific T cells, we used OT-I TCR transgenic, Rag KO mice.

To determine whether OVA-variant antigens are expressed in pancreatic β cells, OT-I mice were crossed to the various RIP-OVA variant mice to generate double transgenic F1 animals (i.e. OT-I TCR x RIP-OVA F1, OT-I TCR x RIP-mQ4H7 F1; OT-I TCR x RIP-mT4 F_1 and OT-I TCR x RIP-mQ4R7 F_1). Surprisingly, double transgenic F_1 mice of all crosses showed high mortality within the first two weeks after birth (89% in OT-I x RIP-mQ4H7; 100% in OT-I x RIP-mT4; 97% in OT-I x RIP-mQ4R7; 64% in OT-I x RIP-OVA) (Fig.1). Blood glucose levels of newborn mice (2-4 day after birth) were elevated in all crossings (OT-I x RIP-mQ4H7 22 mmol/l, OT-I x RIP-mT4 29 mmol/l, OT-I x RIP-mQ4R7 26 mmol/l, OT-I x RIP-OVA 30 mmol/l vs. C57Bl/6 13 mmol/l) indicating severe hyperglycemia. Crossing of OT-I mice to RIP-OVA (that express the soluble form of OVA) or RIP-mOVA (expressing the membrane bound form of OVA) showed similar results (see legend to Fig. 1). From these results, we conclude that all RIP-OVA variant transgenic mice expressed the OVA protein in the β cells of the corresponding RIP-OVA strains. Considering that only a small fraction of mTECs present OVA variant self-peptide to developing T cells, which are present at an extremely high precursor frequency in these F_1 mice, it seems likely that the central tolerance mechanisms were overwhelmed in these mice.

Figure 1

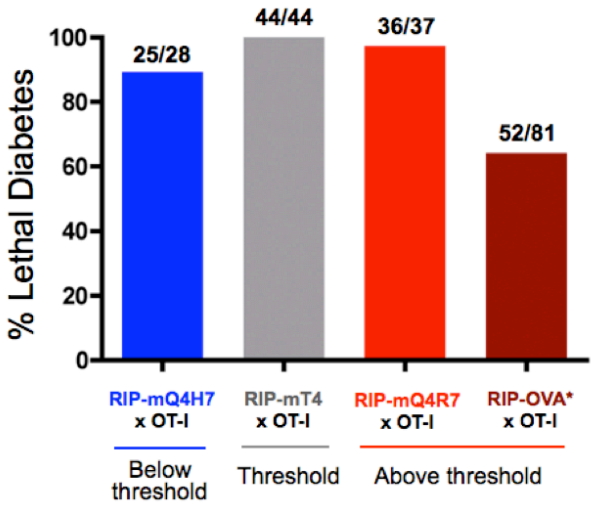

Figure 1. Incidence of Lethal Autoimmune Diabetes in OVA protein variants and OT-I TCR Double Transgenic Mice.
Rag-2-deficient OT-I mice were crossed to RIP-OVA (RIP-OVA shown together with RIP-mOVA) or transgenic mice expressing OVA variant proteins (OVA-Q4R7, OVA-T4, OVA-Q4H7) under the control of the rat insulin promoter (RIP). Mortality was assessed by comparing the number of pups born with the number surviving at 2 weeks. Numbers above the bars indicate number of deceased mice per total mice born. * indicates pooled result of RIP-sOVA (52% mortality, 16/31) and RIP-mOVA (72% mortality, 36/50).

Below threshold T cells comprise a low risk for autoimmunity

Viral and bacterial infections have been used to initiate experimental autoimmunity (Asplin et al., 1982; Ohashi, 1991; Zehn et al., 2009a). Epitopes from pathogens that cross-react with self-antigens can trigger auto-reactive T cells, a phenomenon known as molecular mimicry (Fujinami and Oldstone, 1989; Guilherme et al., 2001). Often, the pathogen-derived antigen has a higher affinity for the TCR on a self-reactive T cell compared to the self-antigen. This results in a full (i.e. high affinity) activation of the weakly auto-reactive T cells, which then attack host cells expressing the lower affinity self-antigen.

To determine whether the affinity of a self-antigen expressed on the pancreatic β cells influences the ability of self-reactive T cells to induce diabetes in the context of molecular mimicry, we transferred OT-I T cells into RIP-variant mice expressing below threshold, threshold, or above threshold OVA variants. Following adoptive transfer, the various RIP-strains were subsequently infected with recombinant Lm expressing the highest affinity OVA epitope (Lm-OVA). Fig. 2A shows that RIP-transgenic mice expressing threshold- (RIP-mT4) and above threshold- OVA variants (RIP-mQ4R7, RIP-OVA) mice developed diabetes when as few as $3x10^3$ OT-I T cells were transferred. This was not the case in the transgenic strain (RIP-mQ4H7) expressing the below threshold variant Q4H7; adoptive transfer of as many as $3x10^5$ OT-I T cells did not result in glucosuria.

Autoimmune diseases can also be initiated by priming auto-reactive T cells with self-antigens. In this case, the affinity of the priming antigen is identical to that of the target antigen. To test the ability of a self-antigen to prime OT-I T cells and initiate autoimmune diabetes, OT-I T cells were transferred into various RIP-transgenic strains followed by immunization with Lm expressing the same OVA variant as is expressed in pancreas (Fig. 2B). Since diabetes induction in RIP-sOVA mice was similar in RIP-mOVA mice, we combined them in one group (RIP-OVA). For a breakdown of the two strains, see legend to Fig. 2. In this homologous self-antigen model we observed that diabetes induction with a low number of OT-I T cells ($3x10^3$ OT-I) in mice expressing above threshold self-antigens (RIP-OVA and RIP-mQ4R7) was very high (100%, 9/9 and 73%, 8/11). Mice expressing the threshold variant, RIP-mT4 required 100 times as many OT-I T cells ($3x10^5$) for diabetes induction. In

contrast, there was no induction of diabetes in RIP-mQ4H7 mice expressing the below threshold OVA variant.

Diabetes induction was not the result of Lm infection itself, since control groups receiving Lm but no OT-I T cells were free of diabetes, throughout all transgenic mouse lines (data not shown). Additionally, previous studies showed that the induction of diabetes is antigen-specific because infection with empty Lm (without OVA protein) did not induce diabetes in RIP-OVA mice (data not shown). These data strongly suggest that in a model where the immunizing and target antigens are identical, only above threshold antigens are able to induce autoimmune diabetes. On the other hand, T cells activated by antigens at- or below- the negative selection threshold exhibit a low potential for induction of autoimmunity.

Figure 2

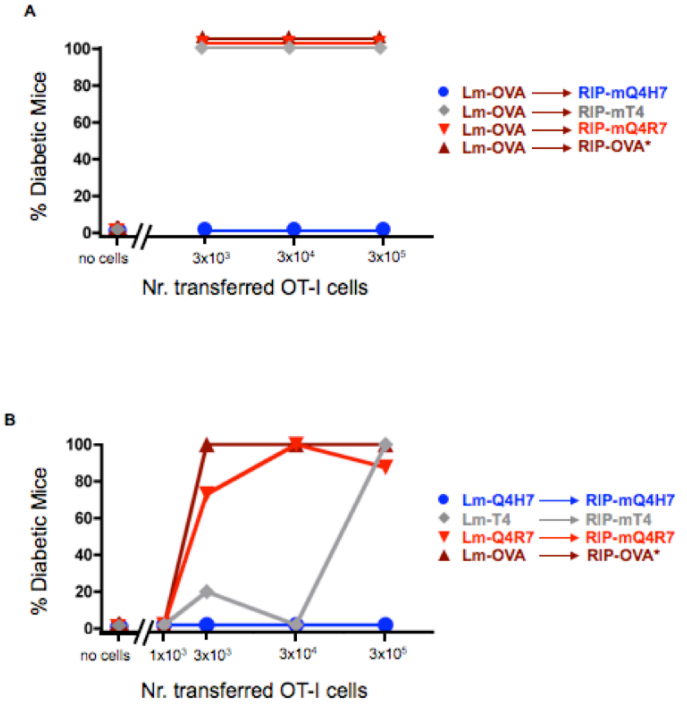

Figure 2. Threshold and below threshold T cells comprise a low risk for autoimmunity.

(A) Immunization with high affinity OVA protein expressed in *Listeria monocytogenes*. Urine glucose was monitored in RIP-OVA and RIP-OVA variant mice injected with the indicated number of OT-I T cells followed by infection with *Listeria monocytogenes* (Lm) expressing high affinity OVA (SIINFEKL, 3000-5000 CFU/mouse).

(B) Immunization with self-antigen expressed in *Listeria monocytogenes*. Mice were injected with the indicated number of OT-I T cells and one day later infected with Lm expressing the appropriate self-antigen. Mice were considered diabetic if urine glucose levels were equal or sustained higher than> 1000 mg/dl. * indicates pooled result of RIP-sOVA (100%, n= 2-5 mice per group) and RIP-mOVA (100%, n= 2-5 mice per group).

Negative selection affinity threshold observed *in vivo*

The high mortality in the breeding of double transgenic mice made it impossible to examine the effect of affinity on deletion of OT-I T cells in detail. Therefore, we generated mixed bone marrow (BM) chimeras, in which a mixture of B6 BM (70%) and OT-I Rg KO BM (30%) was injected into previously irradiated transgenic RIP-variant hosts (Fig. 3A). Additionally, irradiated B6 mice received the same BM mixture as a positive control, where OT-I T cells could develop in the absence of negative selection. Using non-lymphoid cells as a readout, all chimeric mice were similarly reconstituted with OT-I bone marrow (21-33%) (Fig. S2). As readout for negative selection, we assessed the frequency (Fig. 3B) and number (Fig. 3C) of single positive CD8αβ$^+$ OT-I T cells in the lymph nodes of the reconstituted chimeras, 12 weeks after BM transfer. The extent of clonal deletion was calculated in the various RIP-variant chimeras, using the number of OT-I T cells present in OT-I/B6 BM→B6 chimeras as a baseline of OT-I development in the absence of overt negative selection. Clonal deletion of OT-I T cells was not observed in RIP-mQ4H7 (below threshold) or RIP-mT4 (threshold) hosts (Fig. 3B, C); in fact these strains contained slightly increased numbers of OT-Is compared to B6 hosts (Fig. 3C). In contrast, the efficiency of clonal deletion was 91% in RIP-mQ4R7 (above threshold) hosts and >99% efficient in RIP-OVA (highest affinity) hosts (Fig. 3D). Taken together, these data indicate a striking threshold effect that dictates the efficiency of negative selection *in vivo* (Fig. 3D). This is similar to what we observed in fetal thymic organ culture (Daniels et al., 2006).

Figure 3

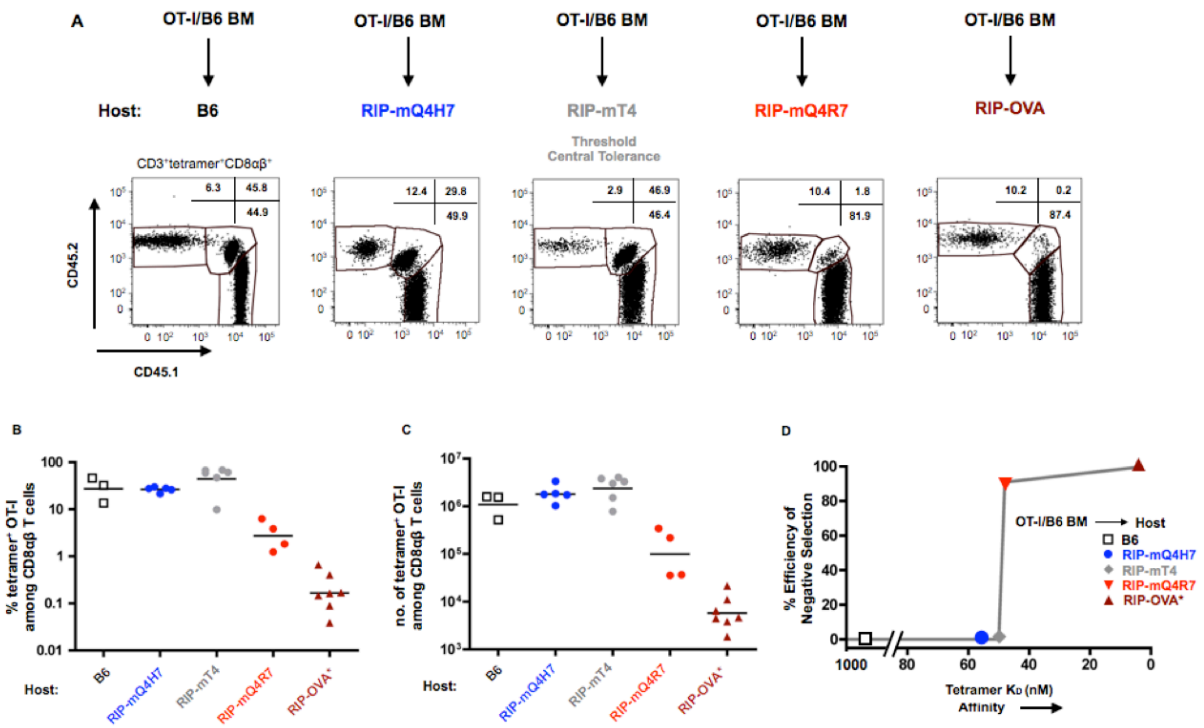

Figure 3. Efficiency of OT-I deletion in OT-I/B6 mixed bone marrow chimeras expressing OVA variant proteins is dependent on antigen affinity.
(A-D) Lethally irradiated RIP-OVA or RIP-OVA variant or B6 mice were injected with a 7:3 mixture of B6 (CD45.1) and OT-I (co-expressing CD45.1 and CD45.2) bone marrow. Flow cytometric analysis of lymphocytes was performed 12-15 weeks after reconstitution. Lymph node (LN) cells were stained with mAbs, CD45.1 and CD45.2 to identify OT-I derived donor (CD45.1/CD45.2), B6 derived donor (CD45.1), remaining host cells (CD45.2). In addition, LN cells were stained with α-CD3 (BD), α-CD8 (eBioscience), α-CD8 (Biolegend) and K^b-OVA tetramers to confirm identity of OT-I T cells.
(A) Representative flow -cytometry plots of OT-I/B6WT mixed bone marrow chimeras are shown. Data show the increasing efficiency of deletion with increasing self-antigen affinity for the OT-I TCR. **(B and C)** OT-I T cells encountering supra-threshold ligands during thymic selection are more completely depleted than T cells encountering threshold or sub-threshold ligands. * indicates pooled result of RIP-sOVA (0.3%, 9537, n= 4) and RIP-mOVA (0.1%, 5156, n= 3) Data show the percentage **(B)** and absolute numbers **(C)** of OT-I T cells within the CD8 T cell compartment. The horizontal bars in B and C represent the geometric mean value of the individual data points. * indicates pooled results of RIP-sOVA (0.3%, 9537, n= 4) and RIP-mOVA (0.1%, 5156, n= 3). Results shown are collected from 6 independent experiments.
(D) Sharp drop in efficiency of negative selection in mice expressing threshold OVA variants in the thymus. The Efficiency of negative selection for each bone marrow chimeric strain relative to chimeras made in B6 hosts (see Experimental Methods for calculation) is plotted versus OT-I affinity for respective K^b-OVA variant antigens.

Phenotype of T cells escaping negative selection

Although negative selection in mice expressing supra-threshold OVA variants is fairly efficient, it fails to eliminate all self-reactive OT-I T cells (Fig. 3C). We wondered whether OT-I T cells escaping negative selection in mice expressing above threshold self-antigens (RIP-mQ4R7 and RIP-OVA), are phenotypically different from OT-I T cells that developed in mice expressing below threshold self-antigens. An elevated expression of CD44 was observed only in RIP-OVA hosts (Fig. 4A). In addition, OT-I T cells surviving negative selection in RIP-OVA chimeras had a slightly elevated expression of CD122 (Fig. 4 A, B). Using CD44, and CD62L expression to define OT-I subpopulations with effector and central memory phenotypes, we found that an increase in the percentage of these two populations was only observed in OT-I T cells maturing through the highest affinity RIP-OVA hosts (Fig. 4A; 4B top panels). The increase of these two memory populations was not correlated with negative selection per se, since negatively selecting RIP-mQ4R7 hosts did not support an increase in their frequency.

Recently, Tsai et al. demonstrated that low-affinity, antigen-specific $CD8^+$ $CD44^+$ $CD122^+$ T cells with suppressive properties could be expanded upon immunization of mice with self-antigen coupled to nano-particles (Tsai et al., 2010). We wondered whether this population could also develop as a consequence of negative selection. Since $CD8^+$ $CD44^+$ $CD122^+$ OT-I T cells were present in all chimeric strains (Fig. 4A; 4B bottom right panel), there was no clear correlation of their presence with negative selection in this experimental model.

Studies of the intestinal mucosa revealed a special type of $TCR\alpha\beta^+$ T cells expressing the atypical CD8αα homodimer (Denning et al., 2007; Latthe et al., 1994). The development of these intraepithelial lymphocytes (IEL) is restricted by classical MHC class I and is driven by the expression of high affinity for self-antigen (Cruz et al., 1998; Poussier et al., 2002).

The CD8αα co-receptor does not efficiently pair with the TCR, allowing for a poor transduction of negative selecting signals (Arcaro et al., 2001; Cawthon et al., 2001). This generates thymocytes, which bypass apoptosis and emigrate to the periphery. However, CD8αα are poor effectors given the weaker function of the CD8αα co-receptor. To confirm whether the presence of peripheral CD8αα OT-I T cells

correlated with self-antigen affinity and negative selection, these populations were identified by flow cytometry in the various chimeric strains (Fig. 4C). Indeed, chimeric mice expressing supra-threshold OVA variants contained markedly increased frequencies of CD8αα OT-I T cells (RIP-mQ4R7 vs. B6 host, p=0.06, RIP-OVA vs. B6 host, p=0.03).

Figure 4

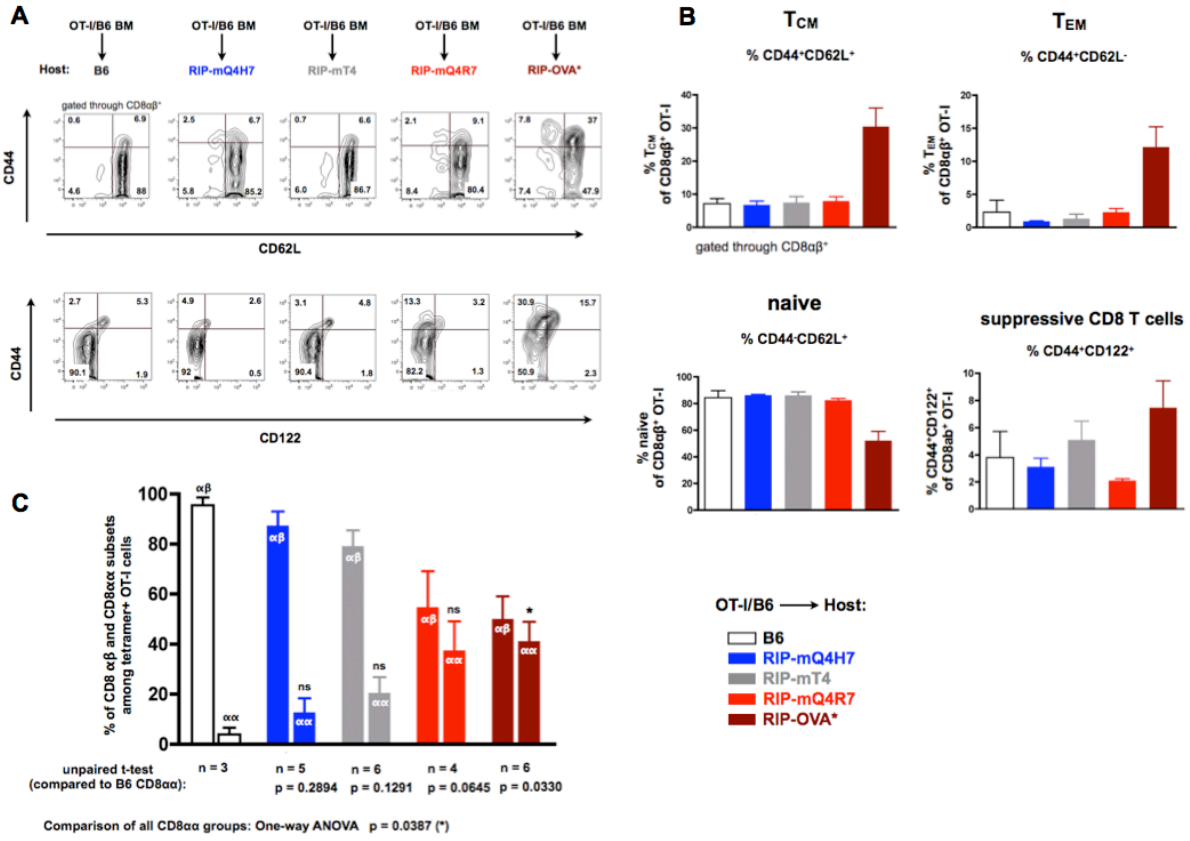

Figure 4. Phenotypic characterization of OT-I T cells in mixed bone marrow chimeras.

(A-D) OT-I T cells escaping negative selection in various bone marrow chimeric mice were phenotypically characterized. **(A-B)** Generation of effector and memory T cells in bone marrow chimeras. Lymphocytes from bone marrow chimeras were stained with mAb specific for CD44, CD62L and CD122. Representative flow-cytometry plots of lymph node cells from bone marrow chimeras are shown. Numbers in the plots depict frequency (%) of cells in each quadrant. **(A)** Expression of CD44, CD62L and CD122 on OT-I T cells in various RIP-OVA-variant bone marrow chimeras.

Representative expression levels of bone marrow chimeras are shown. Colored line represents the surface expression of CD44, CD62L, or CD122 on OT-I T cells while the black line represents the level of expression by polyclonal B6 donor CD8 T cells.

(BC) Analysis of OT-I T_{CM} and T_{EM} compartments in RIP-OVA-variant bone marrow chimeras. The fractions of T_{CM} ($CD44^+CD62L^+$), T_{EM} ($CD44^+CD62L^-$), and naïve ($CD44^-CD62L^+$), and suppressive ($CD44^+CD122^+$), OT-I CD8 $\alpha\beta^+$ cells areis shown. * indicates pooled results of RIP-sOVA (n = 4) and RIP-mOVA (n = 3). **(CD)** OT-I T cells selected on supra-threshold ligands have increased proportion of CD8 OT-I T cells. The percentage of CD8 and CD8 OT-I T cells in each chimeric strain is shown (n = 3 to 7 mice/group). * indicates pooled result of RIP-sOVA and RIP-mOVA (3 mice per group). Unpaired two-tailed Student's t test was performed.

Highest potential for autoimmunity occurs just above the affinity threshold

All chimeric mice were tolerant, as there was no sign of spontaneous autoimmune diabetes. To examine the robustness of self-tolerance in these animals, we challenged each chimeric strain by immunization with the identical self-peptide + LPS (Fig. 5). This experimental setup allowed us to assess the risk of developing autoimmune diabetes when challenged with the same self-antigen used to establish self-tolerance. OT-I/B6 BM → RIP-mQ4H7 chimeric mice immunized with Q4H7 peptide + LPS developed neither glucosuria nor lethal diabetes. The combination of a below threshold immunogen and a below threshold target antigen were insufficient to produce diabetes in spite of the presence of large numbers (~ 10^6) of OT-I T cells in these animals (Fig. 3C). On the other hand, 100% (8/8) OT-I/B6 BM → RIP-mT4 chimeric mice immunized with the threshold peptide, T4 + LPS developed a transient glucosuria (Fig. 5A) that resolved in 50% of these animals (compare gray symbols in Fig. 5A and B). In this model, the threshold ligand can induce autoimmune symptoms, but not always a lethal disease (50%). Considering above threshold self-antigens, a high frequency (86%) of OT-I/B6 BM → RIP-mQ4R7 chimeras immunized with Q4R7 + LPS developed glucosuria and lethal diabetes (Fig. 5A,B). In contrast, not a single (0/10) OT-I/B6 BM → RIP-OVA chimera immunized with OVA peptide + LPS developed glucosuria or lethal diabetes (Fig. 5A,B). In contrast to the RIP-mQ4R7 chimeras, the tolerant state in these highest affinity RIP-OVA chimeras is robust and stable.

To determine whether a difference in the number of regulatory T cells accounted for the difference in diabetes susceptibility between RIP-mQ4R7 and RIP-OVA chimeras, the $CD4^+FoxP3^+$ compartment (derived from B6 donor BM) was identified in all chimeric mice by flow cytometry. All chimeric strains regardless of the affinity of their OVA variant self-antigen, contained a similar frequency (12-18%, data not shown) of $CD4^+FoxP3^+$ regulatory T cells. This was expected since the only difference between the RIP strains is a MHC class I presented epitope. This indicated that the frequency of Tregs cannot explain the dramatic differences between the propensity

of the RIP-mQ4R7 and RIP-OVA chimeras to maintain T cell tolerance and avoid developing autoimmune diabetes.

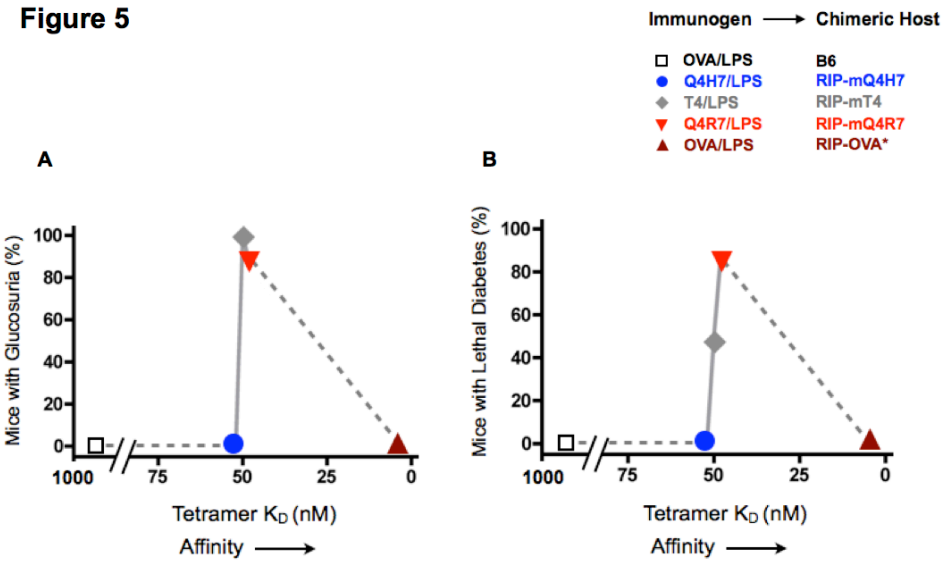

Figure 5. Potential for autoimmunity occurs at or slightly above the affinity threshold for central tolerance.
(A-B) Bone marrow chimeras were generated as described in Figure 3, and then immunized with LPS and the same self-peptide as expressed by the chimeric host. Immunization with self-peptide and LPS followed. Percentage of immunized chimeric mice with **(A)** glucosuria and **(B)** lethal diabetes is shown. Of note, RIP-OVA, RIP-mQ4H7 and C57BL/6 were constantly free of diabetes (urine glucose = 0 mg/dl for up to two weeks following immunization). * indicates pooled results of RIP-sOVA (n = 5) and RIP-mOVA (n = 4).

Relationship between self-antigen affinity and risk of developing autoimmunity

Based on these results, we plotted self-antigen affinity vs. the number of peripheral OT-I cells and color-coded the propensity to develop autoimmune diabetes upon self-antigen challenge (Fig. 6). In Fig. 6A, parameters derived from the four strains of RIP-variant chimeric mice (from Figs. 3 and 5) are plotted. In Fig. 6B, the risk of developing lethal diabetes to self-antigens with a wide variety of affinities and a wide range of self-reactive peripheral T cells was extrapolated based on the following assumptions. Since the risk of developing diabetes in the RIP-mQ4H7 chimeras is close to zero, we assumed that a chimeric mouse expressing a self-antigen of lower affinity compared to Q4H7 or containing lower numbers of peripheral OT-I T cells would also carry a very low risk (~ 0%) of developing this autoimmune disease. Accordingly, the sectors of the graph labeled with a 1 are blue to indicate a very low risk. Mice expressing a self-antigen at the affinity threshold have an intermediate (~ 50%) risk (coded in yellow, sector 2) of developing lethal autoimmunity but only if they contain large numbers (~ $2x10^6$) of OT-Is (Figs. 2B, 5); therefore, we assume that mice carrying smaller numbers of threshold affinity T cells will have a correspondingly low (~ 0%) risk of developing autoimmunity (sectors labeled 3).

In contrast, the risk of developing diabetes is very high (~90%) in RIP-mQ4R7 chimeras, which express the above threshold antigen, Q4R7 and contain ~ 10^5 OT-Is. For this reason, we assumed that chimeric mice expressing an even higher affinity self-antigen and containing ≥ 10^5 OT-Is would also have a very high (≥ 90%) risk of developing this form of autoimmunity. Accordingly, we coded the sector 4 of the graph in red. Finally, RIP-OVA chimeras containing only $5x10^3$ OT-I T cells remain tolerant to a challenge with OVA + LPS. Since their risk of developing autoimmunity is very low, we assumed that chimeric mice containing as few as $5x10^3$ OT-Is and expressing a lower affinity self-antigen would also have a very low risk of developing autoimmune diabetes. For this reason, we colored sector 5 of the graph in blue to indicate a very low risk.

Finally in Fig. 6C, the hypothetical plot in Fig. 6B has been corrected for the effects of central tolerance. Since above threshold self-antigens prevent most but not all

self-reactive thymocytes from entering the periphery, the risk of developing autoimmunity is significantly reduced. As illustrated in Fig. 6C, we propose that the “dangerous” fraction of the peripheral T cell repertoire is concentrated among T cells with a TCR affinity that is just above the negative selection threshold (e.g. the affinity of OT-I for Q4R7). At this affinity, negative selection is incomplete, but the escaping T cells have sufficient affinity to induce autoimmune diabetes in this model system (King et al., 2012). At higher TCR affinities, negative selection is increasingly complete such that the few escaping T cells are incapable of inducing a lethal autoimmune disease.

Figure 6

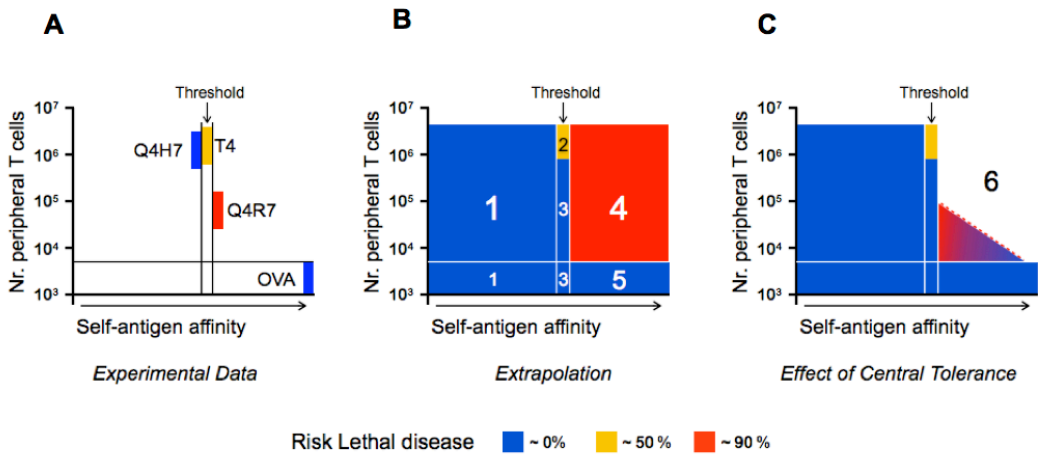

Figure 6. Self-antigen affinity vs. risk of developing autoimmunity
(A) The results from the bone marrow chimeras in Fig. 5 are plotted (self-antigen affinity vs. number of peripheral OT-Is). The negative selection affinity threshold is indicated by an arrow. The risk of developing diabetes following peptide + LPS immunization is indicated for each chimeric strain by a color code (blue: ~ 0% risk; yellow: ~ 50% risk; red: ~ 90% risk).
(B) The data in A have been extrapolated. Chimeric mice expressing OVA variant antigens with an affinity ≤ K^b-Q4H7 or carrying fewer OT-I T cells than RIP-mQ4H7 chimeras (sectors 1) would most likely have a low risk of developing autoimmunity in this model. Chimeric mice expressing a threshold affinity OVA variant antigen are at moderate risk if they contain large numbers of OT-I T cells (sector 2), but are at low risk with lower numbers of OT-Is (sectors 3). Chimeric mice expressing OVA variant antigens with an affinity ≥ K^b-Q4R7 or carrying more OT-I T cells than RIP-mQ4R7 chimeras (sector 4) are predicted to have a high risk of developing autoimmunity. Finally, chimeric mice expressing OVA variant antigens with an affinity ≤ K^b-OVA and carrying fewer OT-I T cells than RIP-OVA chimeras (sector 5) are predicted to be at low risk for autoimmunity.
(C) The extrapolated plot in **(B)** has been corrected for the effects of negative selection. Central deletion removes large numbers of OVA reactive T cells with TCR affinities above the negative selection threshold (sector 6). The deletion of OVA reactive T cells is more efficient with affinities well above the selection threshold (Fig 3D). The net effect is that OVA reactive peripheral T cells with the capacity to cause autoimmune pathology are likely to have a TCR affinity for OVA variant antigen, which is just above the negative selection affinity threshold; in this range of TCR affinities, negative selection is incomplete. In principle, this model could be applied to any self-reactive CD8 T cell.

Discussion

Most autoreactive T cells are depleted from the T cell repertoire (Klein et al., 2009; Stritesky et al., 2012; Zehn and Bevan, 2006). Nevertheless, some self-reactive T cells occasionally escape with the potential to induce autoimmunity in the periphery. To address how self-antigen affinity influences several different steps in the development of autoimmune pathology, we established an experimental system to study T cells that developed in the presence of self-antigens with variable affinity for the OT-I TCR. Additionally, this system allowed us to study the effect of antigen affinity on T cell priming and target destruction.

To verify that OVA-variant antigens are expressed in the β-cells of the pancreas, we crossed OT-I mice to RIP-sOVA, RIP-mOVA or OVA variant mice (OT-I TCR x RIP-sOVA, OT-I TCR x RIP-mOVA or OT-I TCR x mOVA variant). We found that all double transgenic mice develop diabetes, indicating expression of the neo-self antigen in the pancreas. In double transgenic mice expressing Q4H7, T4 or Q4R7 variants, the incidence in diabetes is between 89% and 100%, suggesting that central tolerance mechanisms were overwhelmed in these TCR transgenic (quasi-monoclonal) mice. The frequency of lethal diabetes in double transgenic mice generated from RIP-sOVA or RIP-mOVA parents was 64%, indicating that very high affinity self-antigens more efficiently deplete self-reactive thymocytes, allowing some of the double transgenic progeny to survive. These results are consistent with observations of Hubert et al. They found 88% of RIP-mOVA x OT-I double transgenic mice to develop severe diabetes (Hubert et al., 2011). Similarly, McGargill et al. found that 80% of K14-OVAp/OT-I double transgenic mice, where ovalbumin is expressed under control of the Keratin promoter 14, succumb to a lethal autoimmune disease due to insufficient deletion of developing OT-I cells (McGargill et al., 2002). Nevertheless, 20% of these animals survived.

Based on how few mTECs express any tissue-restricted antigen (Anderson et al., 2002; Derbinski et al., 2001; Liston et al., 2004), it is not surprising that the unphysiologically high numbers of developing self-reactive OT-I thymocytes are insufficiently deleted in these double transgenic animals.

We were somewhat surprised to observe diabetes in double transgenic animals expressing the below threshold antigen Q4H7, since previous studies showed this antigen induces positive selection of OT-I thymocytes (Daniels et al., 2006). Furthermore, immunization with Q4H7 and LPS does not break tolerance in adult RIP-OVA mice receiving adoptively transferred OT-I T cells (King et al., 2012). On the other hand, RIP-OVA mice receiving consecutive immunizations of below threshold antigens expressed in different bacterial or viral vectors can induce diabetes (Enouz et al., 2012). A possible explanation for the spontaneous development of diabetes in OT-I x RIP-mQ4H7 mice is that just after birth, an extremely large number of OT-I T cells likely appear in the periphery before significant numbers of Tregs accumulate. In this relatively lymphopenic environment, it is likely that a sufficient number of OT-I T cells expand by homeostatic division generating CTLs that induce diabetes within the first week of life.

It has been suggested that molecular mimicry plays a role in the development of autoimmunity. A pathogen-specific high affinity T cell response can potentially cross-react on self-antigens of the host, but with lower affinity (Gronski et al., 2004; Hudrisier et al., 2001; Nugent et al., 2000). To specifically address the role of antigen affinity in a mimicry model, OT-I T cells were transferred into RIP-OVA and RIP-mOVA variant mice followed by immunization with Lm-OVA expressing the highest affinity OVA peptide (Fig. 2A). In addition to presenting the OVA peptide, Lm infection also activates the innate immune system, stimulates CD4 T cell help and evokes the release of inflammatory cytokines (Pope et al., 2001). Although the transferred OT-Is were fully activated following Lm-OVA infection, RIP-mQ4H7 mice, expressing the below threshold Q4H7 antigen, did not develop diabetes, indicating that host tissues expressing a below threshold self-antigen are difficult to damage. All other RIP-OVA variant mice including the threshold RIP-mT4 animals efficiently developed lethal diabetes (Fig. 2A). Therefore, under conditions of molecular mimicry (high affinity T cell priming), host tissues expressing threshold or above threshold self-antigens are highly susceptible to autoimmune attack.

Another possible mechanism for the induction of autoimmunity is the priming of host's own peripheral T cells by its' own self-antigens under conditions of inflammation. If the host were to experience an infection of a peripheral organ,

inflammation would generate tissue damage resulting in the release and presentation of self-tissue antigens. These conditions might be able to induce a pathogenic T cell response from a tolerant T cell repertoire, i.e. a repertoire that had survived negative selection. To address the role of antigen affinity in this context, RIP-OVA and RIP-OVA variant mice were adoptively transferred with OT-I cells and infected with Lm expressing the same OVA variant expressed by the self-antigen transgenic mouse (Fig. 2B). Our experiments demonstrated that mice expressing self-antigen above the affinity threshold (RIP-mQ4R7 and RIP-mOVA) are at high risk to develop diabetes, even when low numbers of OT-Is were transferred. In contrast, mice expressing the threshold antigen, T4 required 100 times more transferred OT-I T cells to develop diabetes. Since these numbers of cells are unlikely to be present in a polyclonal mouse, the data indicate that below threshold- and threshold- affinity T cells provide a relatively low risk for the induction of autoimmune diabetes in this model.

The risk of developing autoimmunity also relies on the efficiency of negative selection. Many early studies examining tolerance to very high affinity neo-self antigens give limited insight into tolerance to intermediate and low affinity antigens (Kirberg et al., 1994; Kisielow et al., 1988; Kurts et al., 1998; Ohashi, 1991; von Herrath et al., 1994). More recent studies indicate that tolerance to intermediate and low affinity antigens is inefficient (Enouz et al., 2012; Zehn and Bevan, 2006). To address this issue in a more quantitative manner, we generated mixed OT-I/B6 BM → RIP-OVA variant mice (Fig. 3). Our results (Fig. 3 B-D) indicate that hosts expressing below threshold- or threshold- affinity antigens in radiation resistant thymic epithelial cells do not induce negative selection *in vivo*, while hosts expressing above threshold are highly efficient in preventing OT-I T cells from entering the periphery (RIP-mQ4R7, 91% efficient; RIP-mOVA > 99.5% efficient; RIP-sOVA > 99.4% efficient). This confirms our previous observations based on fetal thymic organ cultures (FTOCs) (Daniels et al., 2006) emphasizing that at the negative selection threshold, a very small (< 2 fold) increase in TCR affinity leads to a striking increase in negative selection (K^b-T4 – 0% negative selection → K^b-Q4R7 – 91% negative selection). Nevertheless, it is worth noting that just above the affinity threshold, negative selection is incomplete.

It is interesting to compare our findings to those of Enouz et al. (Enouz et al., 2012). They developed a new OVA-specific TCR, OT-3, which has a threshold affinity for K^b-OVA, which is comparable to OT-I's affinity for K^b-T4. This was determined by the peptide dose-response of OT-3 for OVA peptide, which is a measure of functional avidity; the actual OT-3 affinity for K^b-OVA might vary. In fact, some experiments indicate that the OT-3 affinity for OVA might be slightly higher than the selection threshold (Fig. 4 in Ref. 23), accounting for the ability of K^b-OVA to induce negative selection of developing OT-3s.

T cells exposed to high affinity ligands during their development can enter diverse lineages (Chang et al., 2007; Hayes and Love, 2006; Hogquist, 2001; Izon et al., 2001; Singer, 2002; Smith-Garvin et al., 2010) and in this context, we examined the phenotype of peripheral OT-I T cells in chimeric RIP-mOVA variant mice. Relative to chimeras made in B6 hosts, the frequency of CD8αα OT-Is increased in chimeras expressing above threshold antigens (Fig 4C). This is likely a result of agonist selection, which has been observed previously (Baldwin et al., 2004; Jordan et al., 2001; Leishman et al., 2002b; Zhou et al., 2004). Given the reduced antigen binding displayed by cells expressing the CD8αα co-receptor (Arcaro et al., 2001; Cawthon et al., 2001), CD8αα thymocytes have an increased chance to evade negative selection. In addition, some peripheral OT-Is in these bone marrow chimeras have a central memory ($CD44^{hi}$, $CD62L^{hi}$) or an effector memory ($CD44^{hi}$, $CD62L^{lo}$) phenotype (Fig. 4 A,B). The proportion (%) of these cells was clearly increased in chimeric mice expressing the highest affinity ligand, OVA (Fig. 4B). We also determined the frequency of $CD8^+CD44^{hi}CD122^{hi}$ cells among peripheral OT-Is, since this cell type has been reported to have suppressive activity, preventing the development of diabetes in NOD mice (Tsai et al., 2010). The frequency of $CD8^+CD44^{hi}CD122^{hi}$ cells did not clearly correlate with the state of tolerance in the chimeric strains. Both OT-I/B6 BM → RIP-mT4 (non-tolerant) and OT-I/B6 BM → RIP-mOVA (tolerant) mice contained a similar percentage of $CD8^+CD44^{hi}CD122^{hi}$ OT-Is (Fig 4 B, bottom right panel). On the other hand, the total number of OT-I T cells varies considerably between these two strains (Fig 3 C). From these data, it is difficult to determine the role of $CD8^+CD44^{hi}CD122^{hi}$ OT-Is in maintaining T cell tolerance in this particular model of diabetes.

To determine the extent of self-tolerance in these chimeric mice, various chimeras were immunized with the same peptide + LPS that they express *in vivo*. This particular experimental design allowed us test the combined effect of self-antigen affinity on the efficiencies of negative selection, T cell priming and target cell lysis (Fig. 5). The highest potential for autoimmunity occurs near the affinity threshold for negative selection (RIP-mT4 chimeras: 50% lethal diabetes; RIP-mQ4R7 chimeras: 86% lethal diabetes). In contrast, the below threshold affinity OT-I/B6 BM → RIP-mQ4H7 chimeras and the highest affinity OT-I/B6 BM → RIP-mOVA chimeras failed to develop diabetes. Despite the absence of negative selection (Fig. 3), the below threshold RIP-mQ4H7 animals are likely protected from developing diabetes due to inefficient T cell priming and poor target cell lysis (Fig. 2). On the other hand, despite the potential for highly efficient T cell priming and target cell lysis (Fig. 2), the highest affinity RIP-mOVA chimeras are likely protected from developing diabetes due to extremely efficient negative selection (Fig. 3). The largest risk of developing autoimmune diabetes in this model is near the negative selection affinity threshold. In this narrow range of TCR affinities, negative selection is leaky and the escaping peripheral T cells can be sufficiently activated by their self-antigen to induce autoimmune pathology.

Based on these results, the risk of developing autoimmune diabetes clearly depends on self-antigen affinity, which affects several important parameters: negative selection, T cell priming and target cell lysis. In Fig. 6, the number of peripheral OT-Is present in the various chimeric strains is plotted against the affinity of the OT-I TCR for the various self-antigens. The tendency for each chimeric strain to develop diabetes following immunization is indicated by a color code (Fig. 6A). In Fig. 6B, we extrapolated the risk of developing autoimmune diabetes to cover a wide range of antigen affinities and numbers of peripheral OT-I T cells, but intentionally left out the effect of negative selection. (See Results for a detailed description). In Fig. 6C, the effect of negative selection is included. The net effect of clonal deletion is to prevent high affinity T cells from entering the periphery (sector 6 labeled in white); if the number of high affinity T cells is sufficiently low, then the individual is at low risk for autoimmune diabetes. The weakness in the system is just above the affinity threshold. At this affinity, negative selection is incomplete, but the escaping

T cells are sufficient in number and affinity to induce autoimmune diabetes in this experimental model (King et al., 2012). We propose that the most 'dangerous' fraction of the peripheral T cell repertoire has an affinity for self-antigens slightly above the negative selection affinity.

Zehn et al. 2009 have convincingly shown that below threshold antigens can induce peripheral CD8 T cells to differentiate into CTLs; moreover, Enouz et al. 2012 demonstrated that low affinity antigens can induce diabetes in RIP-OVA mice. This is dependent on the number of transferred T cells; with limited numbers of transferred OT-3s, diabetes induction requires two immunizations with two different infectious agents expressing the self-antigen. With higher numbers of transferred OT-3s, a below threshold antigen induced experimental diabetes. We agree with the findings of Enouz et al. that under some conditions, below threshold affinity antigens can induce autoimmune diabetes. Our study makes the additional but important point that it is much more difficult to induce autoimmune pathology using a below threshold affinity antigen compared to an above threshold affinity antigen (Figs 2, 5; Ref. 21)

The frequency of diabetes observed in RIP-mT4 and RIP-mQ4R7 chimeras is extremely high (50% and 86%, respectively). This is likely due to the fact that chimeras were reconstituted with a large fraction (30%) of bone marrow stem cells derived from OT-I Rag KO mice, generating a high precursor frequency of self-reactive thymocytes in these animals. The frequency of OT-I like cells present in a polyclonal B6 mouse is on the order of several hundred cells (Moon et al., 2011) and this number is reduced as a consequence of negative selection in mice expressing OVA or OVA variant antigens. Given the low precursor frequency of self-reactive T cells specific for a single self-antigen in polyclonal mice, these animals would likely display a decreased frequency of autoimmune pathology compared to our experimental chimeric mice. This is consistent with the observation that only 3% of the human population has an autoimmune disease of any kind (Cooper et al., 2009).

The other point from our study is that there may be a threshold number of T cells required to induce an autoimmune disease. In OT-I/B6 BM → RIP-OVA chimeras, the 5000 peripheral OT-Is is not sufficient to induce diabetes with peptide / LPS immunization. In adoptive transfer experiments a minimum of 100-300 OT-I cells are

likely required to induce diabetes, when immunized with Lm-OVA (data not shown, Ref. 23). This minimal number of self-reactive T cells might be determined by the type of immunization or by the chronicity of self-antigen exposure. Nevertheless, an individual can probably cope with a small number of high affinity, peripheral T cells and still be considered functionally tolerant, i.e. having a low risk of developing autoimmunity. This would allow negative selection to be less than perfect, which seems to be the case (Moon et al., 2011). This is consistent with recent modeling studies (Butler et al., 2013) which propose that an immune or an autoimmune response requires a minimum number (quorum) of T cells.

Finally, our study highlights the importance of the affinity threshold in establishing T cell tolerance. Our results indicate that thymocytes and their T cell descendants expressing self-reactive TCRs just above the affinity threshold have the highest potential to induce an autoimmune disease.

Materials and Methods

Mice. RIP-OVA mice expressing a soluble form of Ova, and RIP-mOVA, expressing a membrane-bound form of Ova under the control of RIP (Kurts et al., 1997; *Behrens et al., 2004 CGK paper*), OT-I TCR transgenic mice recognizing $K^b/Ova_{257-264}$, and CD45-1 congenic C57BL/6 mice were all obtained from The Jackson Laboratory (Bar Harbor, ME).

Generation of OVA variant transgenic mice. The vector carrying the construct containing OVA (SIINFEKL) and human transferrin receptor (Tfr) was previously described (Gleeson, J. Biol. Chem., 1992) (Teasdale et al., 1992). In brief, the cDNA of Transferrin-Receptor-OVA protein (TfR-OVA) construct was cloned into the pBlue Vector (provided by A. Gleeson). Site directed mutagenesis was used to generate RIP-mQ4H7 (SIIQFEHL), RIP-mT4 (SIITFEKL), and RIP-Q4R7 (SIIQFERL) cDNAs. Similar to the RIP-mOVA construct a 3.2 kB fragment was injected into the pronucleus of fertilized C57BL/6 zygotes and transplanted into NMRI foster mothers. Founder mice were obtained and several founders were backcrossed to C57BL/6 mice.
Double transgenic mice were generated by crossing homozygous OT-I with homozygous OVA or OVA variant transgenic mice. We assed the mortality by comparing the number of F1 pubs born and the number of F1 pubs surviving two weeks after birth.
All animal work was done in accordance with the Federal and Cantonal laws of Switzerland.

Generation of bone marrow chimeric mice. Recipient mice (CD45.2) were lethally irradiated with 900 rad (GammaCell, Best Theratronics, CA). Bone marrow cells were isolated from femurs and tibiae from 5-8 week old B6 (CD45.1) and OT-I Rg KO (CD45.1/2) mice. After red blood cell lysis (RBC lysis Buffer, BioLegend), bone marrow cells were filtered (70-µm cell strainer, BD). To deplete mature T cells, bone marrow cells were stained with anti-CD4, anti-CD8, anti-Thy1.1, anti-Thy1.2 (were obtained from hybridoma supernatants, kindly provided by K.Hafen and G.

Holländer) followed by incubation with complement (Low-Tox-M Rabbit Complement, Cedarlane, CA) at 37°C for 45 minutes. This step was repeated for OT-I bone marrow cells, using anti-CD8 antibody only. A 70% : 30% mixture of B6 and OT-I Rag KO bone marrow cells (3.5 x 10^6 total cells) were injected into irradiated recipient mice. Mice were treated with antibiotics (Nopil, Mepha Pharma AG) for approximately 10 wk after irradiation. The congenic markers CD45.1 and CD45.2 were used to identify the T cells after the reconstitution.

Purification of T cells and adoptive cell transfer. Lymphnode suspensions were obtained by disrupting lymph nodes through a 70-µm nylon cell strainer (BD). Single cell suspensions were counted using a Beckman Coulter Z2 particle counter (Beckman Coulter).

Infections. Recombinant *Listeria monocytogenes* (Lm) expressing the full length OVA protein containing the CD8 epitope SIINFEKL (OVA) or altered ligands Q4R7, T4, or Q4H7 were previously described (Zehn et al., 2009b). Frozen stocks of these strains were grown in brain-heart infusion broth (Thermo Fisher Scientific) to mid log phase. Bacterial numbers were determined by measuring the OD at 600 nm. 5,000 cfu per mouse were injected intravenously in 0.9% NaCl (B.Braun).

Urine and blood glucose measurements. Urine glucose was assessed with test stripes from ACCU-CHEK (DIABUR-TEST 5000, ACCU-CHEK). Mice with sustained urine glucose levels >1000 mg/dl were considered diabetic. Blood samples from the tail vain were read on a Contour blood glucose reader (Bayer). Mice with blood glucose levels >15 mmol/l were considered to be hyperglycemic.

Tetramer, surface antibody staining and flow cytometry.

K^b-OVA (SIINFEKL) tetramers were produced as previously described (Cebecauer et al., 2005; Daniels et al., 2006). Unspecific Fc receptor blocking was performed using purified CD16/32 antibody (BD) for 10 min at 0°C in PBS supplemented with 3% FCS (FACS buffer). After that, cells were washed and K^b-OVA tetramer staining was performed at 0°C in FACS buffer. After 60 min, surface staining was performed (in

the presence of tetramer) for 30 min at 4°C in FACS buffer with the following antibodies: CD45.1-FITC, CD45.1-PE, CD45.2-APC-Cy7, CD8α-biotin, CD4-Alexa700, CD3ε-APC (all from BD) CD8β-PerCP Cy5.5, CD44-APC, CD122-FITC, CD62L-PE-Cy7 (all from BioLegend) and CD8α-PE-Cy7 (eBioscience).

Data analyses. Flow cytometry measurements were performed on a FACS Canto II (BD) machine and the data were analyzed with FlowJo software (Tree Star). Graphs were generated with Prism (GraphPad Software). The horizontal line represents geometric mean. Where indicated, unpaired-t-test and One-way-ANOVA was performed (Prism, GraphPad Software).

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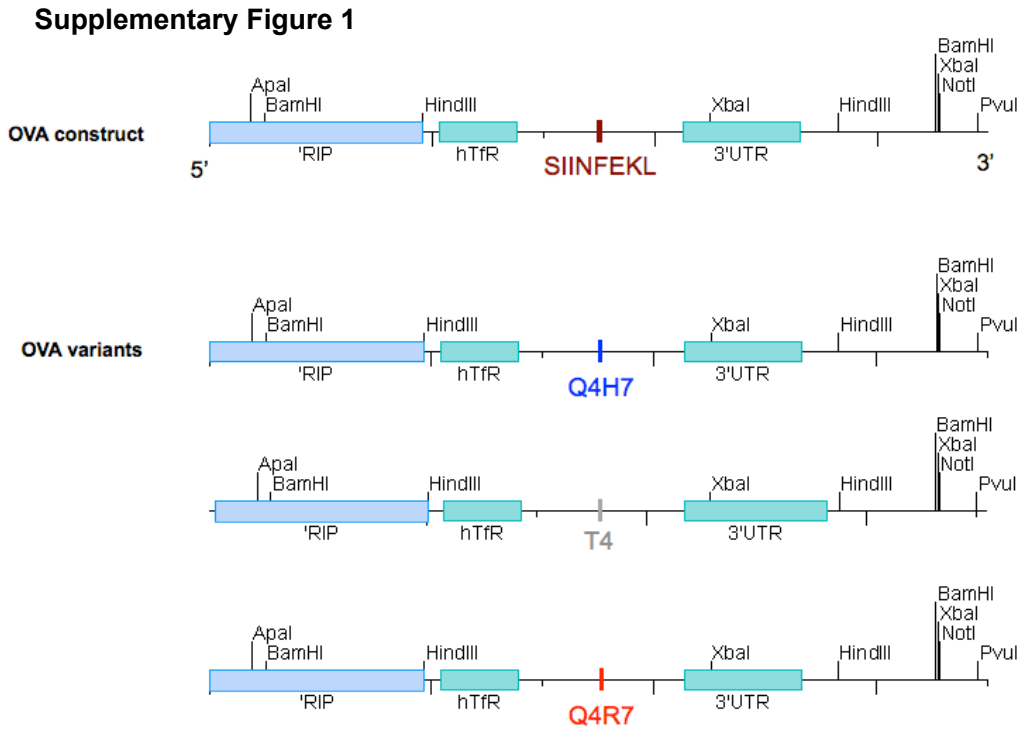
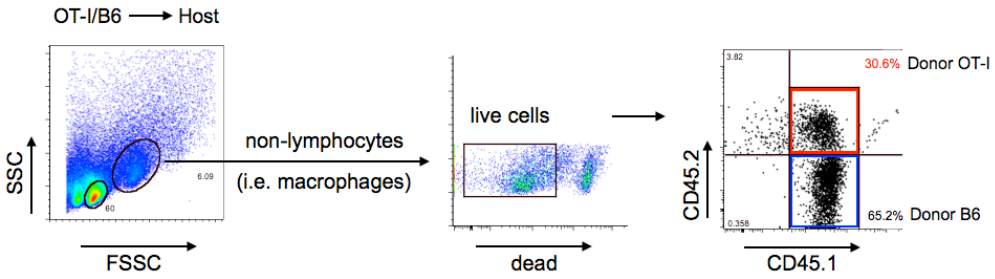


Fig. S1. OVA and OVA variant constructs

The vector carrying the construct containing OVA (SIINFEKL) and human transferrin receptor (Tfr) was previously described (Gleeson, J. Biol. Chem., 1992) (Teasdale, D'Agostaro et al. 1992). In brief, the cDNA of Transferrin-Receptor-OVA protein (TfR-OVA) construct was inserted cloned into the pBlue Vector (provided by A. Gleesonurchased from the laboratory of Gleeson). just before the Hind III site. Site directed mutagenesis was Three different variants of the short region within the OVA cDNA were used to generate RIP-mQ4H7 (SIIQFEHL), RIP-mT4 (SIITFEKL), and RIP-Q4R7 (SIIQFERL) cDNAsrespectively. This was achieved by digesting the vector at the restriction sites Apa I, Not I and Pvu I, allowing a reliable purification of theSimilar to the RIP-mOVA construct a 3.2 kB Apa I, Not I fragment containing the TfR-OVA or OVA variant mutation aimed for injection. The 3.2 kB fragment was injected into the pronucleus of fertilized C57BL/6 zygotes and transplanted into NMRI foster mothers. Founder mice were obtained and selected several founders were backcrossedred to C57BL/6 mice.

Supplementary Figure 2



OT-I/B6 ⟶ Host:	Reconstitution by OT-I BM (Mean%) ± SD	
B6	26 ± 6	n = 3
RIP-mQ4H7	24 ± 5	n = 5
RIP-mT4	33 ± 9	n = 6
RIP-mQ4R7	21 ± 9	n = 4
RIP-sOVA	26 ± 11	n = 4
RIP-mOVA	26 ± 2	n = 3

Fig. S2: Reconstitution by OT-I bone marrow. A 70% : 30% mixture of B6 and OT-I Rag KO bone marrow cells (3.5 x 10^6 total cells) were injected into irradiated recipient mice. The congenic markers CD45.1 and CD45.2 were used to identify the T cells after the reconstitution transfer. Reconstitution by OT-I bone marrow was assed by CD45.1 + frequency in living non-lymphocytes. The table below shows Mean (%) per group.

7.3 Part III, Supplementary Figures

Infiltration of pancreas.
(described in Materials and Methods, Hematoxilin/Eosin staining, page 37)

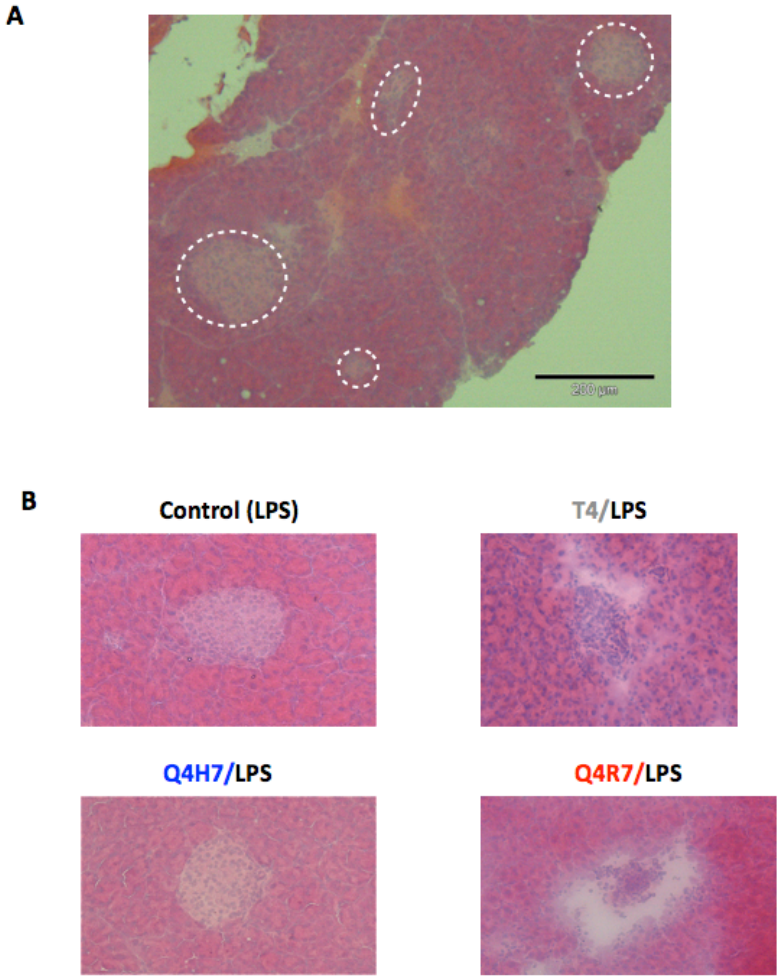


Figure 1. Tissue destruction after immunization with suprathreshold peptide ligands.
(A-B) Mice received $5x10^6$ OT-I T cells followed by immunization with indicated peptide/LPS.
(A) Pancreas section of a control mouse (immunization with LPS). The lighter stained parts are β-islet cells (white dotted line) Image is magnified 10x.
(B) Representative Hematoxilin/Eosin staining on pancreas sections taken 5 days after immunization are shown. Images are magnified 40x.

Infiltration of pancreas.
(described in Materials and Methods, Immunohistochemistry, page 37)

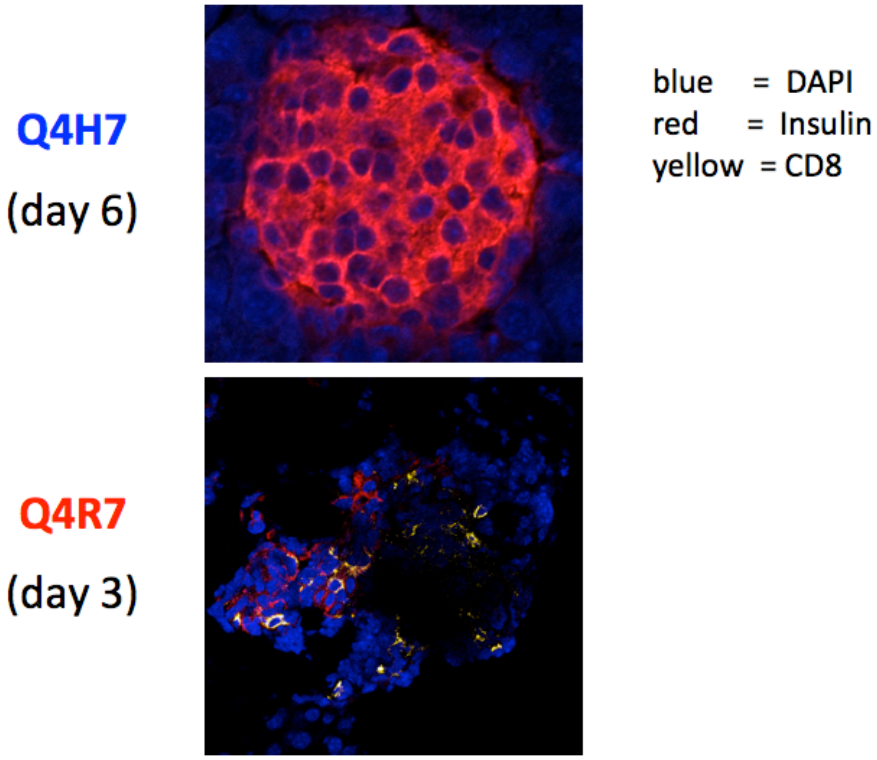


Figure 2. Tissue destruction after immunization with suprathreshold peptide ligands.
Mice received $5x10^6$ OT-I T cells followed by immunization with indicated peptide/LPS. Representative immunohistochemistry for CD8 (yellow), insulin (red), and DAPI (blue) on pancreatic sections taken 6 or 3 days after immunization are shown. Images are magnified 40x.

Incidence of Lethal Autoimmune Diabetes in OVA protein variants and OT-I TCR Double Transgenic Mice.
(discussed in Materials and Methods, Generation of OVA variant transgenic mice, page 35 and in the General Discussion, page 97)

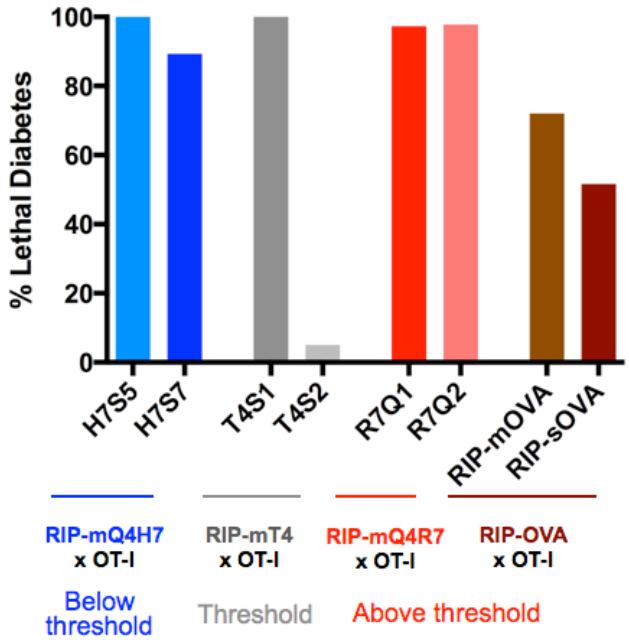


Figure 3. Incidence of Lethal Autoimmune Diabetes in OVA protein variants and OT-I TCR Double Transgenic Mice.
Rag-2-deficient OT-I mice were crossed to RIP-sOVA or RIP-mOVA or transgenic mice expressing OVA variant proteins (RIP-mQ4R7, RIP-mT4, RIP-mQ4H7) under the control of the rat insulin promoter (RIP). From every OVA variant, two founders were generated and crossed to OT-I mice. Mortality was assessed by comparing the number of pups born with the number surviving at 2 weeks. Numbers above the bars indicate number of deceased mice per total mice born.

Threshold and below threshold T cells comprise a low risk for autoimmunity.
(discussed in Materials and Methods, Generation of OVA variant transgenic mice, page 35 and in the General Discussion, page 97)

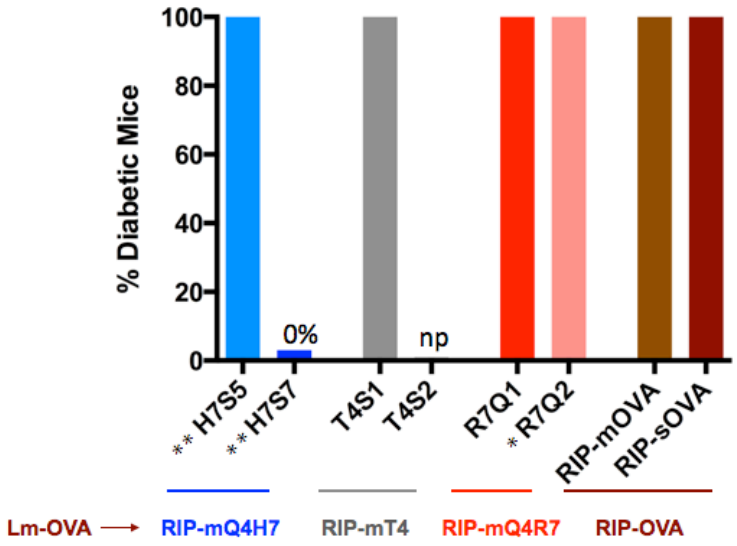


Figure 4. Threshold and below threshold T cells comprise a low risk for autoimmunity.
Immunization with high affinity OVA protein expressed in *Listeria monocytogenes* (Lm-OVA). Urine glucose was monitored of RIP-sOVA, RIP-mOVA and in all RIP-OVA variant founders injected with $3x10^3$ OT-I T cells (* $3x10^5$; ** $3x10^7$ OT-I T cells) followed by infection with Lm-OVA (3000-5000 CFU/mouse). RIP-mOVA (n = 5), RIP-sOVA (n = 4), R7Q1 (n = 3), R7Q2 (n = 3), T4S1 (n = 8), H7S5 (n = 5), H7S7 (n = 10). np means not performed.

Efficiency of OT-I deletion in OT-I/B6 mixed bone marrow chimeras expressing OVA variant proteins is dependent on antigen affinity.
(discussed in Materials and Methods, Generation of OVA variant transgenic mice, page 35)

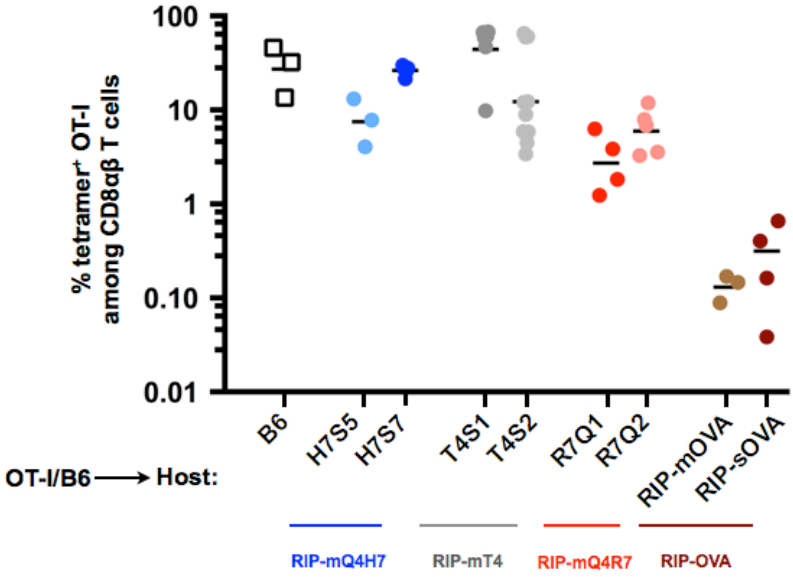


Figure 5 : Efficiency of OT-I deletion in OT-I/B6 mixed bone marrow chimeras expressing OVA variant proteins is dependent on antigen affinity.
Lethally irradiated RIP-OVA or RIP-OVA variant or B6 mice were injected with a 7:3 mixture of B6 (CD45.1) and OT-I (co-expressing CD45.1 and CD45.2) bone marrow. Flow cytometric analysis of lymphocytes was performed 12-15 weeks after reconstitution. T cells from lymphnodes were stained with mAbs, CD45.1 and CD45.2 to identify OT-I derived donor (CD45.1/CD45.2), B6 derived donor (CD45.1), and remaining host cells (CD45.2). In addition, T cells were stained with α-CD3 (BD), α-CD8α (eBioscience), α-CD8β (Biolegend) and K^b-OVA tetramers to confirm identity of OT-I T cells.
Data show the increasing efficiency of deletion with increasing self-antigen affinity for the OT-I TCR. Data show the percentage of OT-I T cells within the CD8 T cell compartment. The horizontal bars represent the geometric mean value of the individual data points.

7.4 General Discussion

What is the origin of autoimmune T cells? To address this question, I used the RIP-OVA mice expressing high affinity OVA and RIP-variant mice expressing OVA variants under the rat insulin promoter (RIP). The unique feature of this system is that OT-I T cells have varying affinity for the antigen expressed in both the thymus and pancreas of RIP-variant mice. Transfer into and priming of OT-I T cells in RIP-OVA mice results in pancreatic β-cell destruction (Kurts et al., 1998), making this a useful model to study the induction of autoimmune disease. However, given the high affinity of the target antigen and other factors, this model gives only limited insight into the natural origins of autoimmune disease. In human autoimmune diabetes, for example, we do not know the natural origins of the disease; what causes the onset of the disease; or how to address the small precursor frequency of self-reactive T cells before the onset of the disease. However, since diabetes is a T cell mediated disease, and infiltration of the pancreas is a hallmark, this is a very good model to study the role of affinity in the generation of autoreactive T cells. Previous studies using neo-self-antigen models (RIP-OVA; RIP-gp33) to study autoimmune disease are more limited than the RIP-variant model presented in these studies, since they used T cells with high affinity for the neo-self antigen (OT-I; gp33 TCR), which is probably unrealistic given that self-antigens are probably of low affinity.

During my PhD studies, I identified three checkpoints, which are regulated by TCR-pMHC affinity during the induction of autoimmune disease. These checkpoints are:

1.) Deletion of self-reactive T cells in the thymus (Central Tolerance),

2.) Activation of self-reactive T cells in the periphery (Priming)

3.) Destruction of self-antigen expressing cells in target tissue by self-reactive T cells

In experiments with bone marrow chimeras, I found that self-reactive T cells are efficiently deleted (91%) if they encounter an antigen with an affinity just above the negative selection threshold. Nevertheless, upon immunization with the same above-threshold self-peptide, 100% of these mice become diabetic. In contrast, T cells that encounter below threshold ligand in the thymus do not undergo negative selection. This finding goes in line with fetal thymic organ cultures demonstrating

that below threshold ligands induced positive selection in double positive thymocytes, whereas above threshold ligands induced negative selection (Daniels et al., 2006; Naeher et al., 2007). Despite the high number of T cells present in the periphery of these mice, immunization with below threshold ligand does not result in autoimmune disease (Results Part II, Fig. 3 and 5). These data highlight the importance of negative selection in preventing the escape and accumulation of high-affinity self-reactive T cells into the periphery. The critical number of autoreactive T cells required to induce autoimmunity remains an open question.

In addition, these findings indicate that the priming of autoreactive T cells in the periphery also depends on TCR affinity (second checkpoint). Indeed, adoptive T cell experiments showed that the critical cell number to induce diabetes is much lower when above-threshold antigens are used for priming compared to below-threshold antigens (Results Part II, Fig. 2B). An underlying mechanism that allows above-threshold antigens to more efficiently activate self-reactive T cells is the induction of asymmetric cell division (Results Part I, Fig. 5-6).

It would be interesting to assess in future experiments if there is a functional difference in OT-I T cells that undergo selection in the presence of high-affinity compared to low-affinity antigen. To address this, RIP-variant mice expressing the below threshold ligand (RIP-Q4H7) could be immunized with Lm-OVA. An alternative approach would be to sort out OT-I T cells from secondary lymphoid organs of chimeric mice and to transfer them into new hosts (RIP-OVA), followed by priming with Lm-OVA.

In some instances, we were able to induce diabetes in response to threshold antigen. For example, the transfer of allogeneic B6.C-H-2 bm12 B cells (Results Part I, Fig. 1B) or OT-II cells provided $CD4^+$ T cell help (data not shown). In double transgenic mice, tolerance was broken even in below threshold antigen expressing mice in the absence of immunization (OT-I x RIP-mT4; OT-I x RIP-mQ4H7; Results Part II, Fig.1). How these circumstances contribute to the breakdown of tolerance needs to be further evaluated. For example, how do CD4 T cells contribute to diabetes in these mice? What is the role of Tregs in this model? Are Tregs required to maintain tolerance, and if so, is there a critical number of Tregs that prevent autoimmunity?

Independent of the affinity of the self-antigen they express, all chimeric mice were tolerant, with the exception of one founder (data not shown). This founder (R7Q2) expresses the above threshold antigen Q4R7 and developed spontaneous diabetes without immunization. Compared to the other founder (R7Q1) negative selection of OT-I T cells in chimeras was a little less efficient (Results part III, Fig.5). It would be interesting to know how spontaneous autoimmune diabetes is induced in these mice.

In conclusion, affinity plays an important role in central and peripheral tolerance. Future therapies should be directed against self-reactive T cells with an affinity at and just above the affinity threshold, since they display the highest risk for the development of autoimmunity.

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Curriculum Vitae

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Academic Education

2009 - present — **Graduate (Ph.D.) Studies in Immunology,**
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PhD thesis "Affinity threshold for establishing and breaking T cell tolerance"

Supervisor: Prof. Ed Palmer

2009 — **Master of Science (MSc) in Molecular Biology**

2008 - 2009 — **Undergraduate Studies in Virology and Immunology,**
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Master thesis "Titration of immunosuppressive drugs and its impact on IFN-g release of polyomavirus BK- and CMV-specific T-cell response *in vitro*"

Supervisor: Prof. Hans H. Hirsch

2008 — **Bachelor of Science (BSc) in Integrative Biology**

2004 - 2008 — **Studies in Biology, University of Basel**

Skills & Competences

Foreign Languages	English, fluent in speaking and writing French, good knowledge in speaking and writing
Teaching Experiences	Organisation of the PhD Club, 2011-2012, Department of Biomedicine, Hebelstrasse 20, Basel, Switzerland
Methods	**Cell Biology and Immunobiology:** Culturing of cell lines, *in vitro* differentiation of bone marrow derived dendritic cells. Cell isolation using magnetic beads, proliferation assays, effector cell function assays, FACS, ELISPOT, histology, IHC, Light- and Confocal-microscopy. **Microbiology:** Culturing of bacteria, purification of genomic DNA from bacteria **Molecular Biology:** PCR, RT-PCR **Animal testing permission CH/EC** LTK Modul I (FELASA Kategorie B) **Transgenic rodent models:** Intravenous and intra-peritoneal injection of mice. Measurement of glucose in urine and blood, bleeding, isolation of lymphocytes from blood, tissue isolation, -processing, -staining and -analysis. Breeding and genotyping of transgenic mice. Generation of mixed bone marrow chimeras. Bacterial infection models.
Computer Skills	Professional knowledge of Mac OSX and MS Windows Professional knowledge of MS Office, Image J, PRISM, FlowJo, Endnote
Continuing Education	Basics in project management 12-13 and 20 may, 2011, University of Basel, Switzerland

Selected Advanced Trainings

Oral Presentation
Workshop Tolerance and Apoptosis
European Congress of Immunology, ECI
5-8 September 2012, Glasgow, Scotland

Poster Presentation
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3-11 September 2011, Hvar, Croatia

Poster Presentation
World Immune Regulation Meeting, WIRM
24-27 March 2011, Davos, Switzerland

Introductory Course in Laboratory Animal Science,
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Publication List

1. Egli, A., **Köhli, S.**, Dickenmann, M. & H.H. Inhibition of polyomavirus BK-specific T-Cell responses by immunosuppressive drugs. Transplantation 88, 1161-1168 (2009)

2. Egli, A., Dumoulin, A., **Köhli, S.**, and Hirsch, H.H. Polyomavirus BK after Kidney Transplantation - Role of Molecular and Immunologic Markers. Trends in Transplantation. 2009; 3(2):085-102

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4. **Koehli, S.**, Naeher D., Galati V., Zehn D., & Palmer, E. Threshold affinity T cells provide the greatest risk for inducing experimental autoimmune diabetes. (Manuscript in preparation)